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(21) International Application Number: PCT/US99/12251 (22) International Filing Date: 2 June 1999 (02.06.99) (30) Priority Data: 60/087,757 2 June 1998 (02.06.98) US 09/177,953 23 October 1998 (23.10.98) US (71) Applicant (for all designated States except US): ISIS PHARMACEUTICALS, INC. [US/US]; 2292 Faraday Avenue, Carlsbad, CA 92008 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): SANGHVI, Yogesh [US/US]; 2169 Wandering Road, Encinitas, CA 92024 (US). MANOHARAN, Muthiah [US/US]; 7634 Reposado Drive, Carlsbad, CA 92009 (US). RAVIKUMAR, Vasulinga, T. [IN/US]; 6606 Vireo Court, Carlsbad, CA 92009 (US). (74) Agents: CALDWELL, John, W. et al.; Woodcock Washburn Kurtz MacKiewicz & Norris LLP, 46th floor, One Liberty Place, Philadelphia, PA 19103 (US).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: ACTIVATORS FOR OLIGONUCLEOTIDE SYNTHESIS		
(57) Abstract The present invention relates to improved methods for the preparation of nucleoside phosphoramidites and oligonucleotides. In one aspect, the methods of the invention are used to prepare phosphitylating reagents using pyridinium salts as activators. In a further aspect, the methods of the invention are used to prepare internucleoside linkages using activators which include at least one pyridinium salt and at least one substituted imidazole. In a further aspect, methods are provided for the preparation of internucleoside linkages between nucleosides having 2'-substituents using imidazolium or benzimidazolium salts as an activator. In a further aspect, methods are provided for the preparation of internucleoside linkages between nucleosides having bioreversible protecting group that confers enhanced chemical and biophysical properties, without exocyclic amine protection, using imidazolium or benzimidazolium salts as an activator.		

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ACTIVATORS FOR OLIGONUCLEOTIDE SYNTHESIS

CROSS REFERENCE TO RELATED APPLICATIONS

The present application claims benefit of U.S. application ser. no. 09/177,953, filed October 23, 1998, and
5 U.S. provisional application no. 60/087,757, filed June 2, 1998, the contents of each of which are hereby incorporated by reference in their entirety.

FIELD OF THE INVENTION

The present invention relates to improved methods
10 for the preparation of oligonucleotides and nucleoside phosphoramidites. More particularly, the methods utilize activators that have certain advantages over conventional activators used in the preparation of nucleoside phosphoramidites, and in their coupling to form oligomers.
15 More specific objectives and advantages of the invention will hereinafter be made clear or become apparent to those skilled in the art during the course of explanation of preferred embodiments of the invention.

BACKGROUND OF THE INVENTION

20 The study of oligonucleotides has become a key area of interest for many reasons including potential uses in therapeutic and diagnostic applications (Agrawal, S., TIBTECH, 1996, 14, 375-382; Marr, J., Drug Discovery Today,

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1996, 1, 94-102; Rush, W., *Science*, 1997, 276, 1192-1193). One of the more interesting applications of oligonucleotides is the ability to modulate gene and protein function in a sequence specific manner. A direct result of studying
5 oligonucleotides including their analogs in variety of applications is the need for large quantities of compounds having high purity. Presently, the synthesis of oligonucleotides and their analogs remains a tedious and costly process. There remains an ongoing need in this area
10 for developing improved synthetic processes that facilitate the synthesis of oligonucleotides.

Phosphoramidites are important building blocks for the synthesis of oligonucleotides. The most commonly used process in oligonucleotide synthesis using solid phase
15 chemistries is the phosphoramidite approach. In a similar process the support used is a soluble support (Bonora et al., *Nucleic Acids Res.*, 1993, 21, 1213-1217). The phosphoramidite approach is also widely used in solution phase chemistries for oligonucleotide synthesis. Deoxyribo-
20 nucleoside phosphoramidite derivatives (Becaucage et al., *Tetrahedron Lett.*, 1981, 22, 1859-1862) have also been used in the synthesis of oligonucleotides.

Phosphoramidites for a variety of nucleosides are commercially available through a myriad of vendors. 3'-O-
25 phosphoramidites are the most widely used amidites but the synthesis of oligonucleotides can involve the use of 5'-O- and 2'-O- phosphoramidites (Wagner et al., *Nucleosides & Nucleotides*, 1997, 17, 1657-1660; Bhan et al., *Nucleosides & Nucleotides*, 1997, 17, 1195-1199). There are also many
30 phosphoramidites available that are not nucleosides (Cruachem Inc., Dulles, VA; Clontech, Palo Alto, CA).

One of the steps in the phosphoramidite approach to oligonucleotide synthesis is the 3'-O-phosphitylation of 5'-O-protected nucleosides. Additionally, exocyclic amino

groups and other functional groups present on nucleobase moieties are normally protected prior to phosphitylation. Traditionally phosphitylation of nucleosides is performed by treatment of the protected nucleosides with a

5 phosphitylating reagent such as chloro-(2-cyanoethoxy)-N,N-diisopropylaminophosphine which is very reactive and does not require an activator or 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite (bis amidite reagent) which requires an activator. After preparation the nucleoside 3'-

10 O-phosphoramidite is coupled to a 5'-OH group of a nucleoside, nucleotide, oligonucleoside or oligonucleotide.

The activator most commonly used in phosphitylation reactions is 1H-tetrazole. There are inherent problems with the use of 1H-tetrazole, especially

15 when performing larger scale syntheses. For example, 1H-tetrazole is known to be explosive. According to the material safety data sheet (MSDS) 1H-tetrazole (1H-tetrazole, 98%) can be harmful if inhaled, ingested or absorbed through the skin. The MSDS also states that 1H-

20 tetrazole can explode if heated above its melting temperature of 155°C and may form very sensitive explosive metallic compounds. In addition, 1H-tetrazole is known to Hence 1H-tetrazole requires special handling during its storage, use, and disposal.

25 Aside from its toxicity and explosive nature 1H-tetrazole is acidic and can cause deblocking of the 5'-O-protecting group and can also cause depurination during the phosphitylation step of amidite synthesis (Krotz et al., *Tetrahedron Lett.*, 1997, 38, 3875-3878). Inadvertent

30 deblocking of the 5'-O-protecting group is also a problem when chloro-(2-cyanoethoxy)-N,N-diisopropylaminophosphine is used. Recently, trimethylchlorosilane has been used as an activator in the phosphitylation of 5'-O-DMT nucleosides with bis amidite reagent but this reagent is usually

35 contaminated with HCl which leads to deprotection and

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formation of undesired products (Dabkowski, W., et al. *Chem. Comm.*, 1997, 877). The results for this phosphitylation are comparable to those for 1H-tetrazole.

Activators with a higher pKa (i.e., less acidic)
5 than 1H-tetrazole (pKa 4.9) such as 4,5-dicyanoimidazole (pKa 5.2) have been used in the phosphitylation of 5'-O-DMT thymidine (Vargeese, C., *Nucleic Acids Res.*, 1998, 26, 1046-1050).

A variety of activators have been used in the
10 coupling of phosphoramidites in addition to 1H-tetrazole. 5-Ethylthio-1H-tetrazole (Wincott, F., et al., *Nucleic Acids Res.* 1995, 23, 2677) and 5-(4-nitrophenyl)-1H-tetrazole (Pon, R.T., *Tetrahedron Lett.*, 1987, 28, 3643) have been used for the coupling of sterically crowded ribonucleoside
15 monomers e.g. for RNA-synthesis. The pKa's for these activators are 4.28 and 3.7 (1:1 ethanol:water), respectively. The use of pyridine hydrochloride/imidazole (pKa 5.23 (water)) as an activator for coupling of monomers was demonstrated by the synthesis of a dimer (Gryaznov,
20 S.M., Letsinger, L.M., *Nucleic Acids Res.*, 1992, 20, 1879). Benzimidazolium triflate (pKa 4.5 (1:1 ethanol:water)) (Hayakawa et al., *J. Org. Chem.*, 1996, 61, 7996-7997) has been used as an activator for the synthesis of oligonucleotides having bulky or sterically crowded
25 phosphorus protecting groups such as aryloxy groups. The use of imidazolium triflate (pKa 6.9 (water)) was demonstrated for the synthesis of a dimer in solution (Hayakawa, Y.; Kataoka, M., *Nucleic Acids and Related Macromolecules: Synthesis, Structure, Function and*
30 *Applications*, September 4-9, 1997, Ulm, Germany). The use of 4,5-dicyanoimidazole as an activator for the synthesis of nucleoside phosphoramidite and several 2'-modified oligonucleotides including phosphorothioates has also been reported (Vargeese, *supra.*).

- 5 -

Another disadvantage to using 1H-tetrazole is the cost of the reagent. The 1997 Aldrich Chemical Company catalog lists 1H-tetrazole at over ten dollars a gram for 98% material. The 99+% pure material lists for over forty
5 seven dollars per gram. This reagent is used in excess of the stoichiometric amount of nucleoside present in the reaction mixture resulting in considerable cost especially during large scale syntheses.

The solubility of 1H-tetrazole is also a factor in
10 the large scale synthesis of phosphoramidites, oligonucleotides and their analogs. The solubility of 1H-tetrazole is about 0.5 M in acetonitrile. This low solubility is a limiting factor on the volume of solvent that is necessary to run a phosphitylation reaction. An
15 activator having higher solubility would be preferred to allow the use of minimum volumes of reactions thereby also lowering the cost and the production of waste effluents. Furthermore, commonly used 1H-tetrazole (0.45 M solution) for oligonucleotide synthesis precipitates 1H-tetrazole when
20 the room-temperature drops below 20 °C. Thus, blocking the lines on the automated synthesizer.

Due to ongoing clinical demand (See, for example, Crooke et al., *Biotechnology and Genetic Engineering Reviews*, 1998, 15, 121-157) the synthesis of
25 oligonucleotides and their analogs is being performed utilizing increasingly larger scale reactions than in the past. One of the most common processes used in the synthesis of these compounds utilizes phosphoramidites that are routinely prepared and used in conjunction with an
30 activator. There exists a need for phosphitylation activators that poses less hazards, are less acidic, and less expensive than activating agents that are currently being used, such as 1H-tetrazole. This invention is directed to this, as well as other, important ends.

SUMMARY OF THE INVENTION

In one aspect, the present invention presents improved methods for preparing phosphitylated compounds comprising the steps of:

- 5 providing a compound having a hydroxyl group;
 reacting said compound with a phosphitylating reagent in the presence of a pyridinium salt in a solvent under conditions of time, temperature and pressure effective to yield said phosphitylated compound.

- 10 In some preferred embodiments of the invention, the compound having a hydroxyl group is a nucleoside, preferably a 5'-protected nucleoside having a 3'-hydroxyl group. In further preferred embodiments, the compound is a nucleoside dimer having a 3' or 5'-hydroxyl group. In still
15 further preferred embodiments, said compound is a nucleoside having a 5' or 2' hydroxyl group.

In further preferred embodiments, the compound having a free hydroxyl group is an oligonucleotide or oligonucleotide analog having a 3' or 5' hydroxyl group.

- 20 In some preferred embodiments of the invention, the phosphitylating reagent is bis amidite reagent (2-cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite), bis(N,N-diisopropylamino)-2-methyltrifluoroacetylaminomethoxyphosphine or bis(N,N-diisopropylamino)-2-diphenyl-
25 methylsilylethoxyphosphine.

In further preferred embodiments of the invention, the pyridinium salt is pyridinium hydrochloride, pyridinium trifluoroacetate or pyridinium dichloroacetate.

- In further preferred embodiments of the invention,
30 the solvent is dichloromethane, acetonitrile, ethyl acetate, tetrahydrofuran or a mixture thereof.

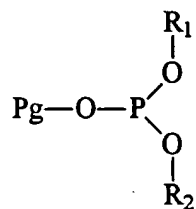
In further preferred embodiments, the activator is bound to a solid support. In Still further preferred embodiments, the activator is a polyvinyl pyridinium salt.

- 35 In a further aspect, the present invention

- 7 -

provides improved methods for the preparation of intersugar linkages. In preferred embodiments, the methods of the invention are used in the preparation of oligonucleotides via standard solid phase oligonucleotide regimes.

- 5 In some preferred embodiments, the present invention presents methods for the preparation of a compound of Formula I:



I

- 10 wherein:

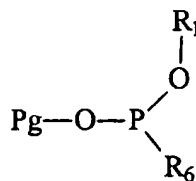
R_1 is a mononucleoside or an oligonucleotide;

R_2 is a nucleoside linked to a solid support, or an oligonucleotide linked to a solid support;

Pg is a phosphorus protecting group;

- 15 comprising:

providing a phosphoramidite of Formula II:



II

- wherein R_6 is $-\text{N}(\text{R}_7)_2$ wherein R_7 is alkyl having from one to
20 about six carbons; or R_7 is a heterocycloalkyl or heterocycloalkenyl ring containing from 4 to 7 atoms, and having up to 3 heteroatoms selected from nitrogen, sulfur, and oxygen;

and reacting said phosphoramidite with a hydroxyl

- 8 -

group of a nucleoside linked to a solid support, or an oligonucleotide linked to a solid support;

said reaction being performed in the presence of an activating reagent, said activating reagent comprising at least one pyridinium salt and at least one substituted imidazole.

Also provided in accordance with the present invention are methods for the preparation of an oligonucleotide comprising the steps of:

providing a 3'-mononucleoside phosphoramidite or 3'-oligonucleotide phosphoramidite; and

reacting said 3'-mononucleoside phosphoramidite or 3'-oligonucleotide phosphoramidite with the 5'-hydroxyl of a nucleoside, nucleotide, or oligonucleotide in the presence of an activating reagent;

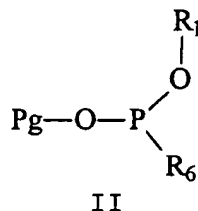
said activating reagent comprising at least one pyridinium salt and at least one substituted imidazole.

In some preferred embodiments, the 3'-mononucleoside phosphoramidite or oligonucleotide phosphoramidite is reacted with the 5'-hydroxyl of a solid-support bound nucleoside, nucleotide or oligonucleotide.

In further preferred embodiments of the foregoing methods of the invention, the oligonucleotide comprises phosphorothioate intersugar linkages.

The present invention also provides synthetic methods comprising:

providing a phosphoramidite of Formula II:



30

wherein

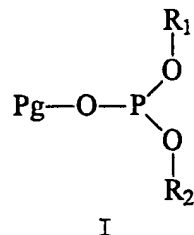
- 9 -

R_1 is a mononucleoside or an oligonucleotide;

Pg is a phosphorus protecting group;

R_2 is $-N(R_7)_2$ wherein R_7 is alkyl having from one to about six carbons; or R_7 is a heterocycloalkyl or
 5 heterocycloalkenyl ring containing from 4 to 7 atoms, and having up to 3 heteroatoms selected from nitrogen, sulfur, and oxygen; and

reacting said phosphoramidite with a hydroxyl group of a nucleoside linked to a solid support, or an
 10 oligonucleotide linked to a solid support, to form a compound of Formula I:



said reaction being performed in the presence of
 15 an activating reagent, said activating reagent comprising at least one pyridinium salt and at least one substituted imidazole; and

oxidizing or sulfurizing said compound to form a compound of Formula III:

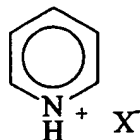


wherein Q is O or S , with S being preferred.

In some preferred embodiments of the foregoing methods, the substituted imidazole is 1-methylimidazole.

25 In further preferred embodiments of the foregoing methods, the pyridinium salt has the formula

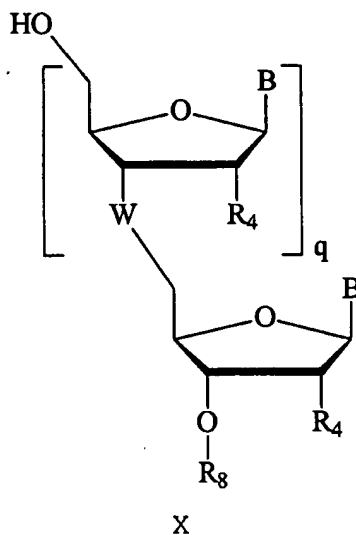
- 10 -



where X^- is trifluoroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br , ^-O -trifluorosulfonyl, hexafluorophosphate or tetrafluoroborate, with trifluoroacetate being preferred.

5 In a further aspect of the invention, synthetic methods are provided comprising:

providing a compound of Formula X:



10 wherein:

B is a nucleobase;

R_8 is H, a hydroxyl protecting group, or a linker connected to a solid support;

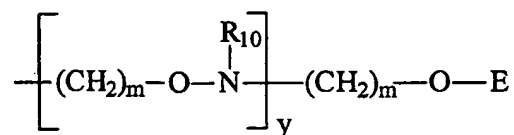
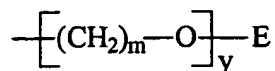
15 W is an optionally protected internucleoside linkage;

q is 0 to about 50;

R_4 is H, F, O-R, S-R or N-R(R_{10});

R is H, a protecting group, or has one of the formulas:

- 11 -



where

each m is independently from 1 to 10;

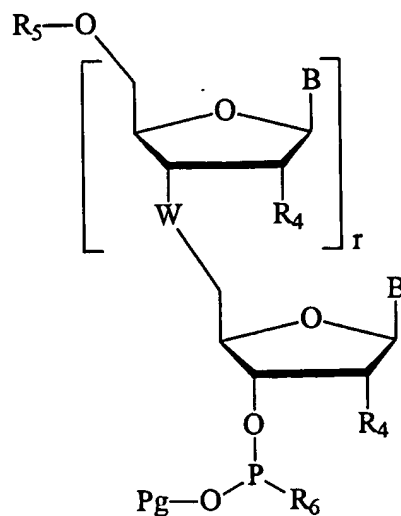
y is from 0 to 10;

- 5 E is H, a hydroxyl protecting group, C₁-C₁₀ alkyl, N(R₁₀)(R₁₁) or N=C(R₁₀)(R₁₁); substituted or unsubstituted C₁-C₁₀ alkyl, C₂-C₁₀ alkenyl, C₂-C₁₀ alkynyl, wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto
- 10 residues;
- each R₁₀ or R₁₁ is, independently, H, substituted or unsubstituted C₁-C₁₀ alkyl, C₂-C₁₀ alkenyl, C₂-C₁₀ alkynyl, wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto
- 15 residues; alkylthioalkyl, a nitrogen protecting group, or R₁₀ and R₁₁, together, are a nitrogen protecting group or wherein R₁₀ and R₁₁ are joined in a ring structure that can include at least one heteroatom selected from N and O;

or R is -CH₂-CH₂-O-CH₂-CH₂-N(R₁₀)(R₁₁);

- 20 reacting the compound of Formula X in the presence of an activating reagent with a compound of Formula XI:

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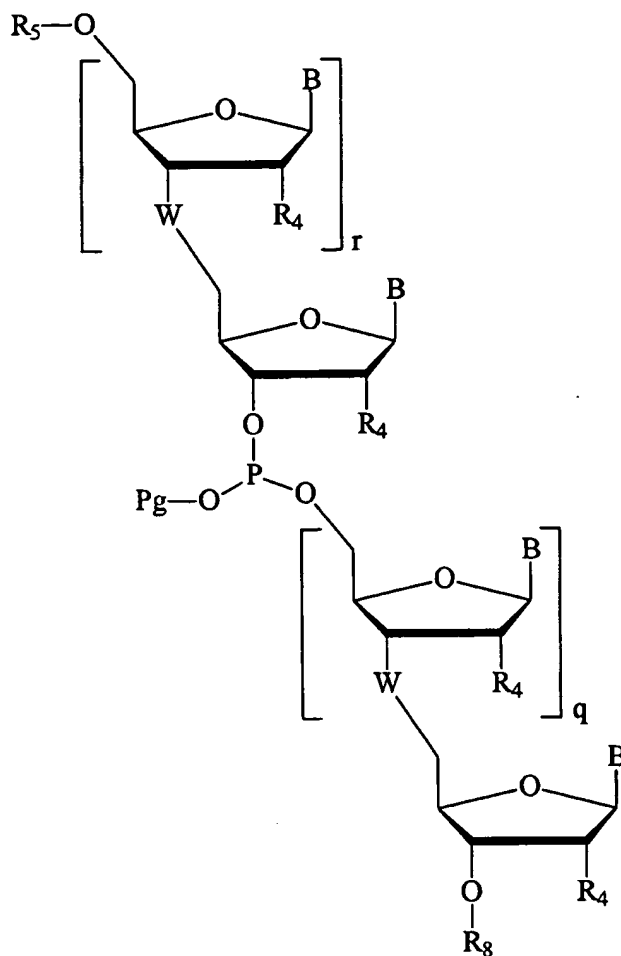
XI

where r is 0 to about 50;

5 R_5 is a hydroxyl protecting group;

R_6 is $-N(R_7)_2$ wherein R_7 is alkyl having from one to about six carbons; or R_7 is a heterocycloalkyl or heterocycloalkenyl ring containing from 4 to 7 atoms, and having up to 3 heteroatoms selected from nitrogen, sulfur,
 10 and oxygen;

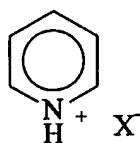
to form a compound of Formula XII:



XII

wherein said activating reagent comprises at least one pyridinium salt and one substituted imidazole.

5 In some preferred embodiments, the pyridinium salt has the formula:



where X^- is trifluoroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br , ^-O -trifluorosulfonyl, hexafluorophosphate, or
 10 tetrafluoroborate, with trifluoroacetate being preferred.

In further preferred embodiments, the substituted

imidazole is 1-methylimidazole.

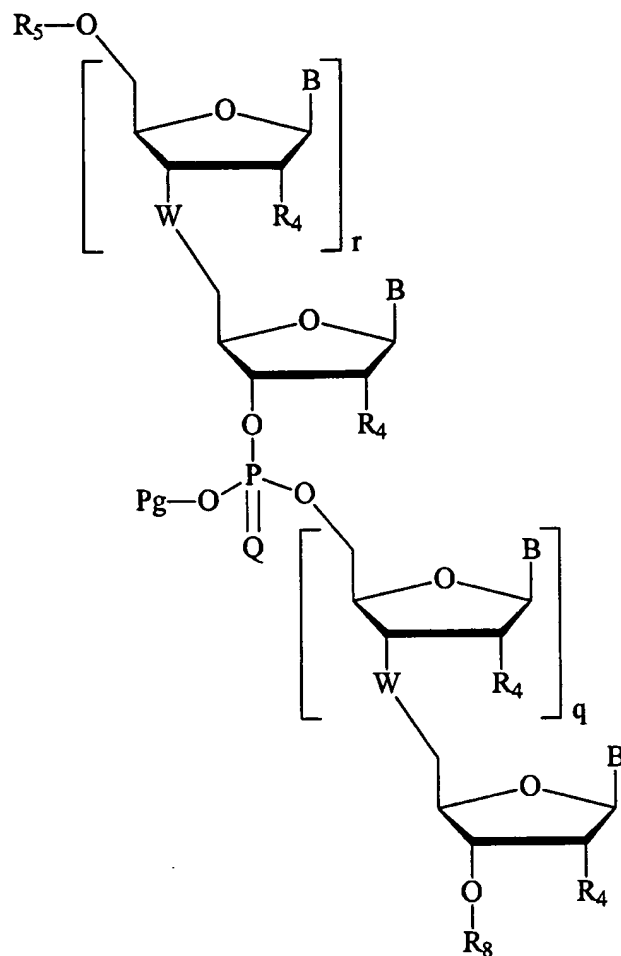
In some preferred embodiments, R_8 is a linker connected to a solid support.

In further preferred embodiments, R_4 is $-O-R$
5 wherein R has the formula $-[(CH_2)_m-O]_y-E$; m is 2, y is 1;
and E is CH_3 , $-N(R_{10})(R_{11})$, or $-CH_2-CH_2-N(R_{10})(R_{11})$.

In further preferred embodiments, r is 0. In
still further preferred embodiments, R_6 is diisopropylamino.

Preferably, P_g is $-CH_2CH_2CN$, $-CH_2CH=CHCH_2CN$, para-
10 $CH_2C_6H_4CH_2CN$, $-(CH_2)_{2-5}N(H)COCF_3$, $-CH_2CH_2Si(C_6H_5)_2CH_3$, or
 $-CH_2CH_2N(CH_3)COCF_3$. with $-CH_2CH_2CN$ being more preferred.

Some preferred embodiment of the methods further
comprising oxidizing or sulfurizing the compound of Formula
XII to form a compound of Formula XIII:



XIII

where Q is O or S, with S being preferred.

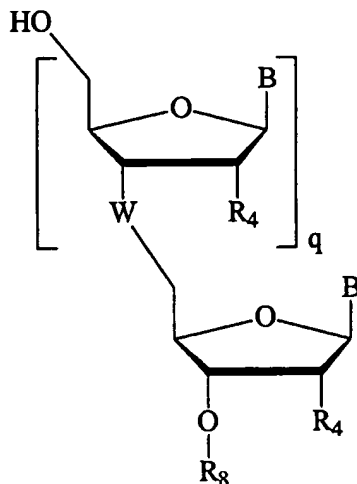
Some further preferred embodiments of the methods further comprising a capping step, which is preferably performed prior to oxidation.

Some further preferred embodiments further comprising the step of cleaving the oligomeric compound to produce a further compound of formula X.

10 In a further aspect of the invention, methods are provided for the preparation of internucleoside linkages between nucleosides having 2'-substituents, using an activating reagent that is preferably an imidazolium triflate. In some preferred embodiments, these methods
15 comprise:

- 16 -

providing a compound of Formula X:



X

5 wherein:

B is a nucleobase;

R₈ is H, a hydroxyl protecting group, or a linker connected to a solid support;

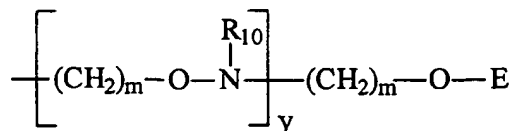
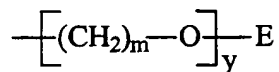
10 W is an optionally protected internucleoside linkage;

q is 0 to about 50;

R₄ is H, F, O-R, S-R or N-R(R₁₀);

R is H, a protecting group, or has one of the formulas:

15



where

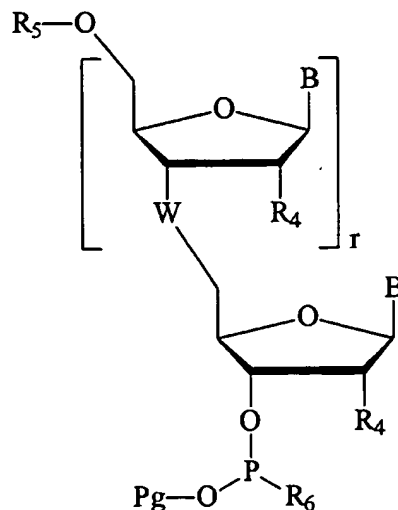
each m is independently from 1 to 10;

y is from 0 to 10;

E is H, a hydroxyl protecting group, C₁-C₁₀

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- alkyl, $N(R_{10})(R_{11})$ or $N=C(R_{10})(R_{11})$; substituted or unsubstituted C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto
- 5 residues; and
- each R_{10} or R_{11} is, independently, H, substituted or unsubstituted C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto
- 10 residues; alkylthioalkyl, a nitrogen protecting group, or R_{10} and R_{11} , together, are a nitrogen protecting group or wherein R_{10} and R_{11} are joined in a ring structure that can include at least one heteroatom selected from N and O;
- or R is $-CH_2-CH_2-O-CH_2-CH_2-N(R_{10})(R_{11})$;
- 15 provided that R_{14} is not H or OH;
- reacting the compound of Formula X in the presence of an activator with a compound of Formula XI:



XI

20

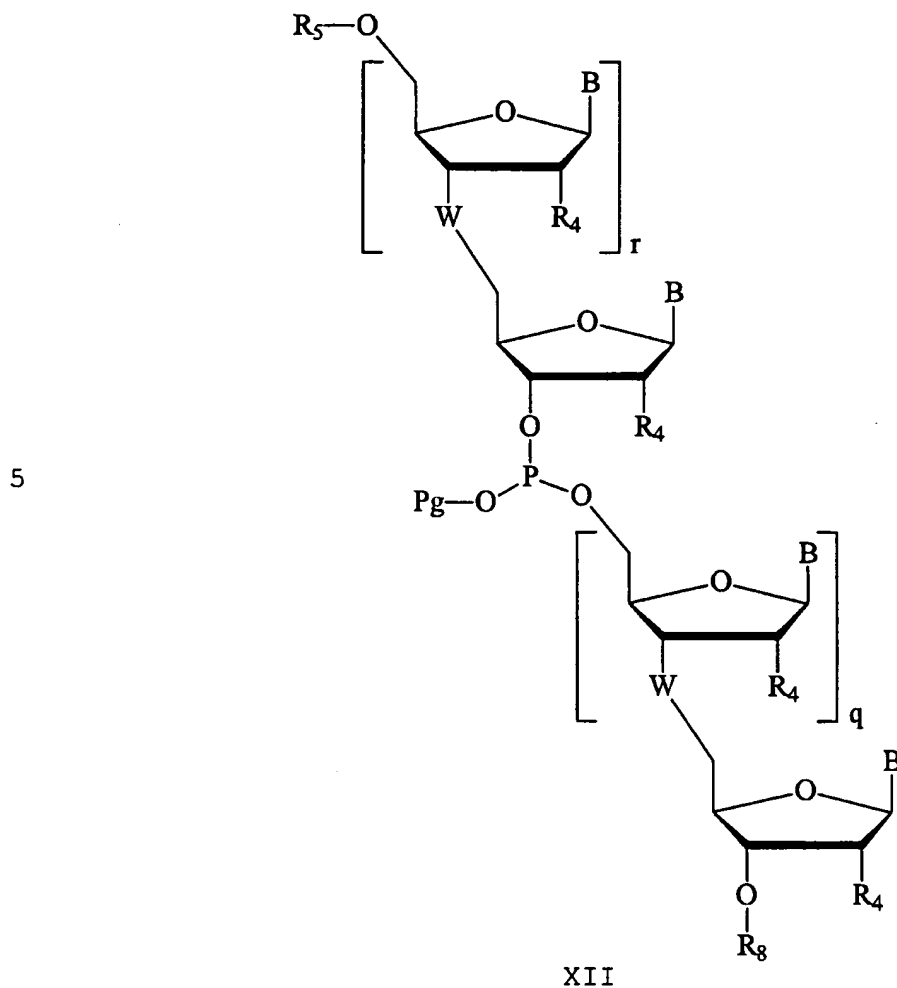
where r is 0 to about 50;

R_5 is a hydroxyl protecting group;

R_6 is $-N(R_7)_2$ wherein R_7 is alkyl having from one to about six carbons; or R_7 is a heterocycloalkyl or

heterocycloalkenyl ring containing from 4 to 7 atoms, and having up to 3 heteroatoms selected from nitrogen, sulfur, and oxygen;

to form a compound of Formula XII:



wherein the activator has the formula G^+U^- ,

where

G^+ is selected from the group consisting of
10 pyridinium, imidazolium, and benzimidazolium; and

U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate, triflate, hydrochloride, trifluoroacetate, dichloroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br , and ^-O -trifluorosulfonyl.

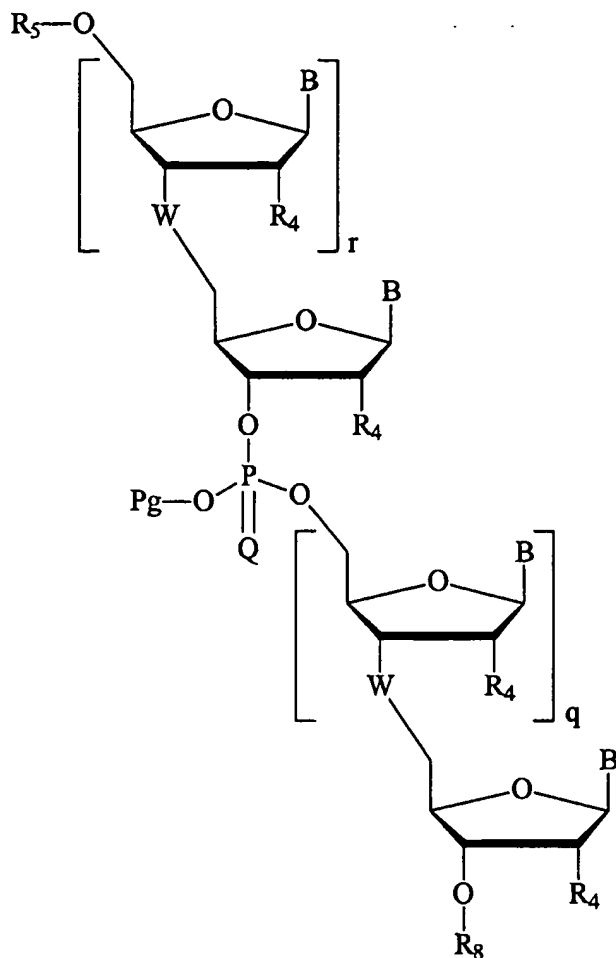
Preferably, the activator is imidazolium triflate.

In some preferred embodiments, R_8 is a linker connected to a solid support. In further preferred embodiments, R_4 is $-O-R$ wherein R has the formula $-[(CH_2)_m-O]_y-E$; m is 2, y is 1; and E is CH_3 , $-N(R_{10})(R_{11})$, or $-CH_2-CH_2-N(R_{10})(R_{11})$.

In further preferred embodiments, r is 0. In still further preferred embodiments, R_6 is diisopropylamino.

Preferably, P_g is $-CH_2CH_2CN$, $-CH_2CH=CHCH_2CN$, para-
10 $CH_2C_6H_4CH_2CN$, $-(CH_2)_{2-5}N(H)COCF_3$, $-CH_2CH_2Si(C_6H_5)_2CH_3$, or $-CH_2CH_2N(CH_3)COCF_3$. with $-CH_2CH_2CN$ being more preferred.

Some further preferred embodiments further comprise oxidizing or sulfurizing the compound of Formula XII to form a compound of Formula XIII:



XIII

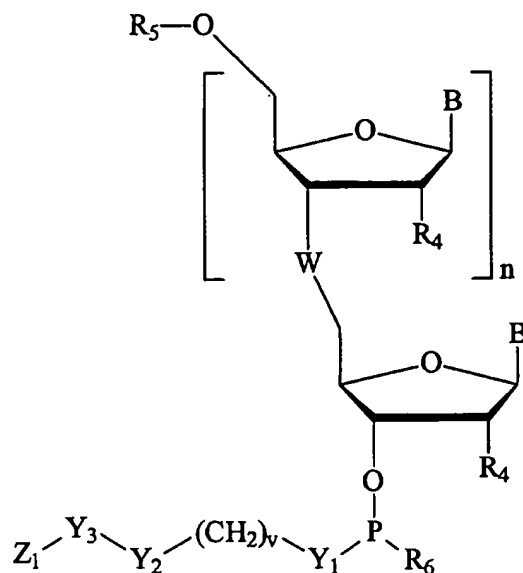
where Q is O or S, with S being preferred.

Some further preferred embodiments of the methods
 5 further comprising a capping step, which is preferably
 performed prior to oxidation.

Some further preferred embodiments further
 comprising the step of cleaving the oligomeric compound to
 produce a further compound of formula X.

10 In a further aspect of the invention, synthetic
 methods are provided comprising:
 providing a compound of Formula XX:

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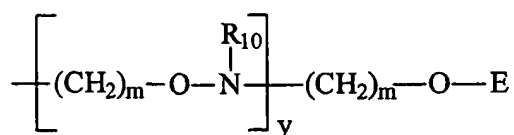
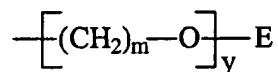


XX

wherein:

 R_4 is H, F, O-R, S-R or N-R(R_{10});

5 R is H, a protecting group, or has one of the formulas:



where

each m is independently from 1 to 10;

10 y is from 0 to 10;

E is H, a hydroxyl protecting group, C_1 - C_{10} alkyl, $N(R_{10})(R_{11})$ or $N=C(R_{10})(R_{11})$; substituted or unsubstituted C_1 - C_{10} alkyl, C_2 - C_{10} alkenyl, C_2 - C_{10} alkynyl, wherein the substitutions are selected from one or several

15 halogen, cyano, carboxy, hydroxy, nitro and mercapto residues; and

each R_{10} or R_{11} is, independently, H, substituted or

unsubstituted C₁-C₁₀ alkyl, C₂-C₁₀ alkenyl, C₂-C₁₀ alkynyl, wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto residues; alkylthioalkyl, a nitrogen protecting group, or R₁₀ and R₁₁, together, are a nitrogen protecting group or wherein R₁₀ and R₁₁ are joined in a ring structure that can include at least one heteroatom selected from N and O;

or R is -CH₂-CH₂-O-CH₂-CH₂-N(R₁₀)(R₁₁);

R₅ is a hydroxyl protecting group;

10 Z₁ is aryl having 6 to about 14 carbon atoms or alkyl having from one to about six carbon atoms;

Y₁ is O or S;

Y₂ is O or S;

Y₃ is C(=O) or S;

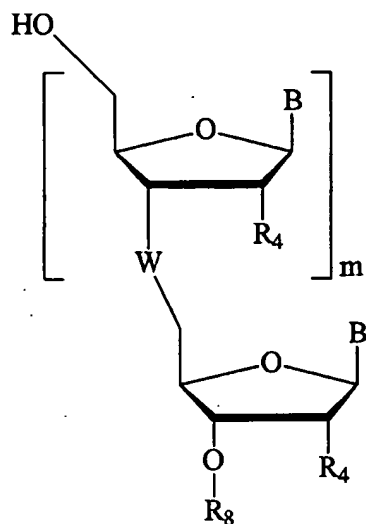
15 v is 2 to about 4;

B is a nucleobase;

R₆ is -N(R₇)₂ wherein R₇ is alkyl having from one to about six carbons; or R₇ is a heterocycloalkyl or heterocycloalkenyl ring containing from 4 to 7 atoms, and
20 having up to 3 heteroatoms selected from nitrogen, sulfur, and oxygen;

reacting said compound of Formula XX with a compound of Formula XXI:

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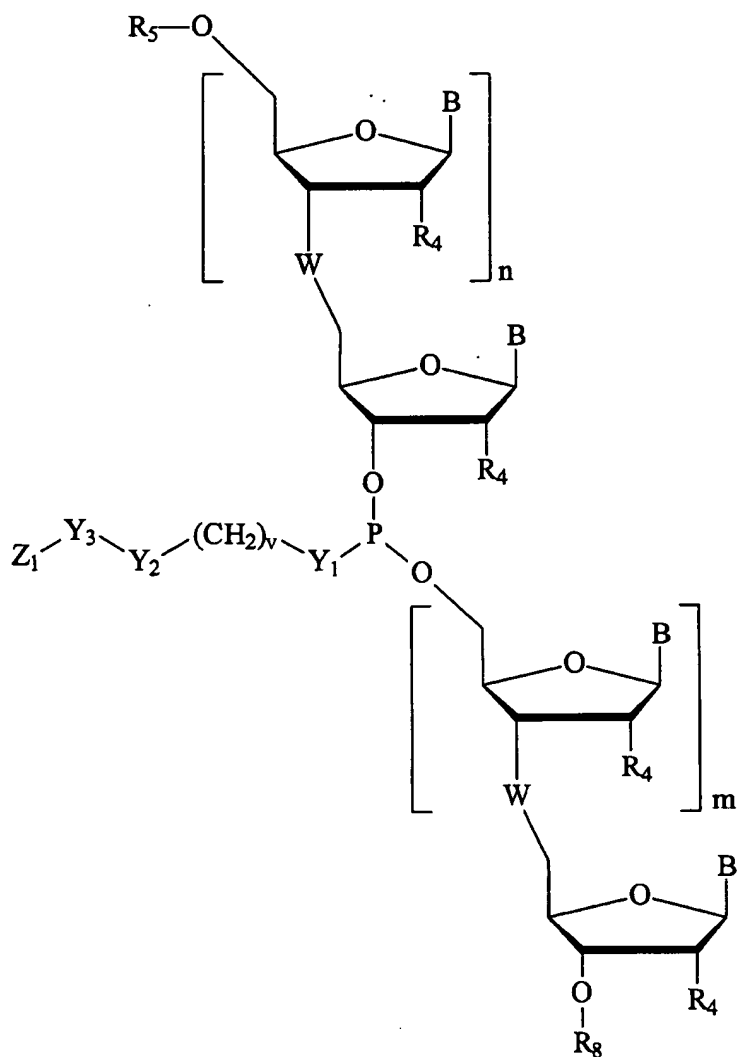


XXI

wherein:

- R_8 is H, a hydroxyl protecting group, or a linker
5 connected to a solid support;
in the presence of an activator to form a compound
of Formula XXII:

- 24 -



XXII

wherein the activator has the formula G^+U^- ,

where

- 5 G^+ is selected from the group consisting of pyridinium, imidazolium, and benzimidazolium; and
- U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate, triflate, hydrochloride, trifluoroacetate, dichloroacetate, ^-O -mesyl,
- 10 ^-O -tosyl, ^-Br , and ^-O -trifluorosulfonyl;
- or said activator is a substituted imidazolium triflate.

Preferably, the activator is imidazolium triflate.

- 25 -

In some preferred embodiments, v is 2; and Y_3 is $C(=O)$. In further preferred embodiments, Z is methyl, phenyl or *t*-butyl, with *t*-butyl being preferred.

In some preferred embodiments, n is 0. In further
5 preferred embodiments, R_8 is a linker to a solid support.

In some preferred embodiments, Y_1 and Y_2 are each O. In other preferred embodiments, Y_1 and Y_2 are each S. In still further preferred embodiments, Y_1 is O and Y_2 is S.

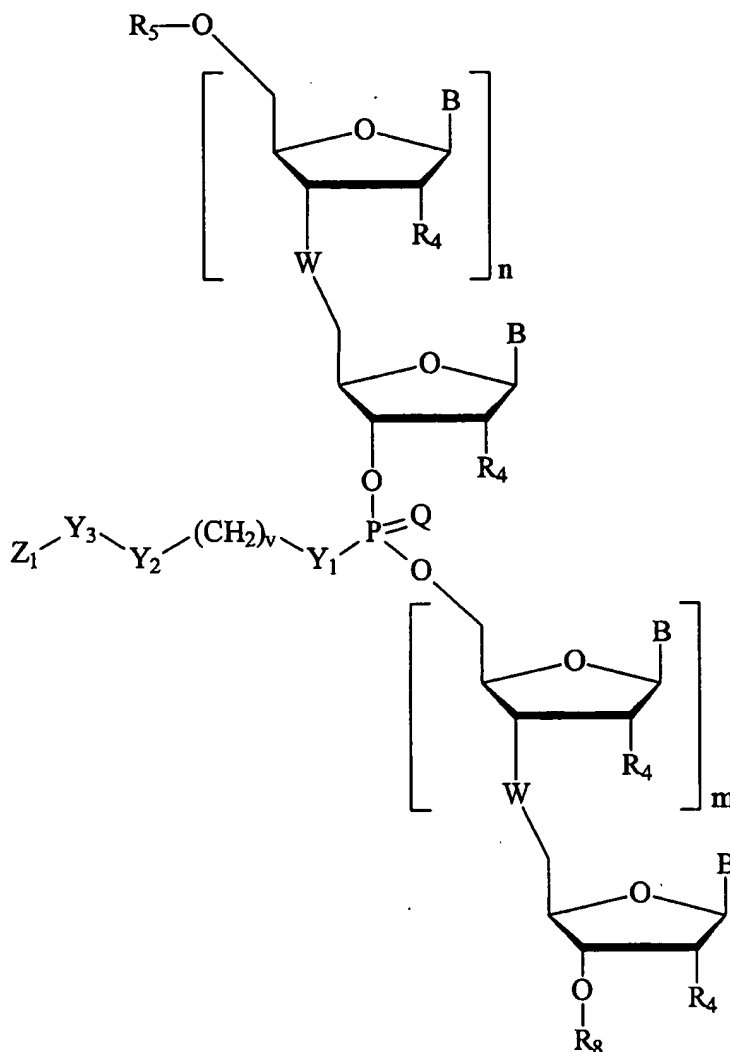
Preferably, each R_7 is isopropyl.

10 In some preferred embodiments, n is 0; R_3 is H, R_6 is diisopropylamino; Y_1 is O; Y_2 is S; and Z is methyl or *t*-butyl, with *t*-butyl being preferred.

In some preferred embodiments of each of the foregoing methods, each constituent nucleobase "B" is devoid
15 of exocyclic amine protection.

Preferably, W is an optionally protected phosphodiester, phosphorothioate, phosphorodithioate, or alkyl phosphonate internucleotide linkage.

Some preferred embodiments of the foregoing
20 methods further comprise oxidizing or sulfurizing the compounds of Formula XXII to form a compound of Formula XXIII:



XXIII

where Q is O or S.

Some further preferred embodiments of the methods further comprising a capping step, which is preferably performed prior to oxidation.

Some further preferred embodiments further comprising the step of cleaving the oligomeric compound to produce a further compound of formula XXI.

In some preferred embodiments, G^+ is pyridinium and U^- is hexafluorophosphate or tetrafluoroborate, with hexafluoroborate being preferred.

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In further preferred embodiments, G^+ is imidazolium or benzimidazolium and U^- is selected from the group consisting of triflate, hydrochloride, trifluoroacetate, dichloroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br ,
5 and ^-O -trifluorosulfonyl.

In other preferred embodiments, G^+ is imidazolium or benzimidazolium and U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate, and triflate.

10 In further preferred embodiments, G^+ is imidazolium or benzimidazolium and U^- is selected from the group consisting of hydrochloride, trifluoroacetate, dichloroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br , and ^-O -trifluorosulfonyl.

15 In still further preferred embodiments, G^+ is imidazolium and U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate, triflate, hydrochloride, trifluoroacetate, dichloroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br , and ^-O -trifluorosulfonyl.

20 In still further preferred embodiments, U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate, and triflate.

In further preferred embodiments, U^- is selected from the group consisting of hydrochloride,
25 trifluoroacetate, dichloroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br , and ^-O -trifluorosulfonyl.

In further preferred embodiments, G^+ is benzimidazolium and U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate, triflate,
30 hydrochloride, trifluoroacetate, dichloroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br , and ^-O -trifluorosulfonyl.

In further preferred embodiments, G^+ is benzimidazolium and U^- is hexafluorophosphate, tetrafluoroborate, or triflate.

35 In further preferred embodiments, G^+ is

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benzimidazolium and U⁻ is selected from the group consisting of hydrochloride, trifluoroacetate, dichloroacetate, ⁻O-mesyl, ⁻O-tosyl, ⁻Br, and ⁻O-trifluorosulfonyl.

In some preferred embodiments, the activator is
5 substituted or unsubstituted imidazolium triflate, with unsubstituted imidazolium triflate being preferred.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a scheme showing intermediates and products in a series of phosphitylation reactions according
10 to the invention.

Figure 2 is a list of activating reagents suitable for use in the present invention.

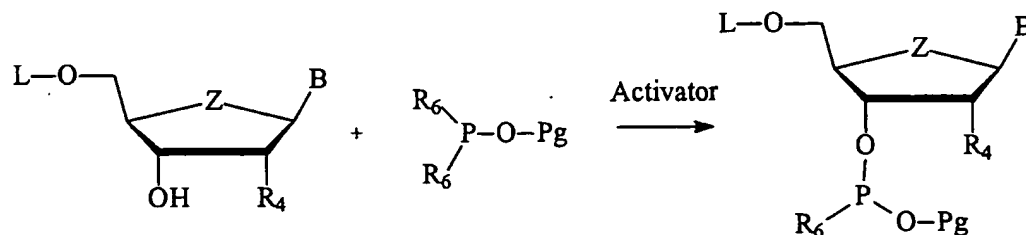
Figure 3 is a list of activating reagents suitable for use in the present invention.

15 Figure 4 is an electropherogram comparing the efficiency of tetrazole activator and pyridinium trifluoroacetate / 1-methylimidazole activator.

DESCRIPTION OF PREFERRED EMBODIMENTS

The present invention describes improved methods
20 for, *inter alia*, phosphitylating compounds having a free ("unblocked") hydroxyl group. In some preferred embodiments, the compound to be phosphitylated is a mononucleoside, an oligonucleotide, or analog thereof.

A large number of compounds are amenable to the
25 improved process of the present invention. A general scheme utilizing some preferred starting materials is illustrated below:



wherein

L is a hydroxyl protecting group, a nucleotide, a nucleoside, an oligonucleotide or and oligonucleoside;

5 Z is O, S, CH₂ or NR₁₀;

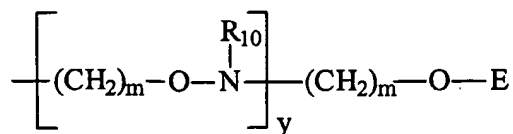
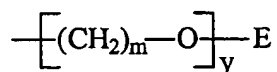
B is a nucleobase or a modified nucleobase;

Pg is a phosphorus protecting group that is preferably -CH₂CH₂CN,

-CH₂CH=CHCH₂CN, para-CH₂C₆H₄CH₂CN, -(CH₂)₂₋₅N(H)COCF₃,
 10 -CH₂CH₂Si(C₆H₅)₂CH₃, or -CH₂CH₂N(CH₃)COCF₃;

R₄ is H, F, O-R, S-R or N-R(R₁₀);

R is H, a protecting group, or has one of the formulas:



15 where

each m is independently from 1 to 10;

y is from 0 to 10;

E is H, a hydroxyl protecting group, C₁-C₁₀ alkyl, N(R₁₀)(R₁₁) or N=C(R₁₀)(R₁₁); substituted or
 20 unsubstituted C₁-C₁₀ alkyl, C₂-C₁₀ alkenyl, C₂-C₁₀ alkynyl,
 wherein the substitutions are selected from one or several
 halogen, cyano, carboxy, hydroxy, nitro and mercapto
 residues; and

- 30 -

each R_{10} or R_{11} is, independently, H, substituted or unsubstituted C_1 - C_{10} alkyl, C_2 - C_{10} alkenyl, C_2 - C_{10} alkynyl, wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto residues; alkylthioalkyl, a nitrogen protecting group, or R_{10} and R_{11} , together, are a nitrogen protecting group or wherein R_{10} and R_{11} are joined in a ring structure that can include at least one heteroatom selected from N and O; or R is $-CH_2-CH_2-O-CH_2-CH_2-N(R_{10})(R_{11})$.

10 The initial step in the phosphitylation scheme illustrated above is the activation of the phosphorus atom of the phosphitylating reagent via protonation. The activator donates a proton to the phosphorus atom of the phosphitylating reagent (i.e., a P^{III} compound having at
15 least one phosphorus/oxygen bond) thereby activating the reagent. The activation involves formation of a salt with the corresponding anion of the activator. When the phosphitylating reagent is activated the phosphorus atom undergoes nucleophilic attack by a free hydroxyl group
20 displacing a diisopropylamino group which forms a salt with the anion of the activator. As depicted above the free hydroxyl group is a 3' hydroxyl group but the attacking nucleophile could alternatively be a 5' hydroxyl group (Wagner, T., and Pfleiderer, W., *Nucleosides & Nucleotides*,
25 1997, 16, 1657-1660) or a 2' hydroxyl group (Bhan et al., *Nucleosides & Nucleotides*, 1997, 16, 1195-1199). The nucleophilic attack results in the formation of a stable phosphoramidite ($P^{(III)}$) compound.

In addition to phosphitylation of 3' hydroxyl
30 positions of nucleosides or larger oligomeric structures the present invention is also amenable to phosphitylation of 5', 2', and 1' hydroxyl positions. The present process is also amenable to compounds other than nucleosides. All that is required is that the compound have an unblocked hydroxyl
35 group and be inert to the reaction conditions of

phosphitylation, or, for example, be rendered inert to the reaction conditions by addition of appropriate protecting groups if necessary. There are numerous examples in the literature of phosphitylation of non-nucleosidic compounds
5 such as for example: alkyl groups (Filippov et al., *Nucleosides & Nucleotides*, 1997, 16, 1403-1406); cyclohexoses (Schlienger et al., *Nucleosides & Nucleotides*, 1997, 16, 1325-1329); peptide nucleic acid (Vinayak et al., *Nucleosides & Nucleotides*, 1997, 16, 1653-1656); macrocyclic
10 ligands (Wagner et al., *Nucleosides & Nucleotides*, 1997, 17, 1789-1792), European Patent Application no. EP 0 816 368 A1, entitled "Chemical Phosphorylation of Oligonucleotides and Reactants used therefor, filed July 2, 1997, published January 7, 1998.

15 In preferred embodiments, the methods of the present invention use pyridinium salts as activators during the synthesis of phosphoramidites. Thus, the methods of the invention possess significant advantages over conventional phosphitylation processes. For example, the activators of
20 the present invention can be generated *in situ* by mixing equal molar amounts of the base pyridine and an acid such as for example HCl, CF₃COOH, CHCl₂COOH or CF₃SO₃H (trifluoromethylsulfonic acid). The *in situ* preparation of activators is quick, easy and provides significant benefit in the
25 performance synthesis of phosphoramidites on a large scale. Particularly, at the oligonucleotide manufacturing site, where pyridine and CHCl₂COOH both are used as synthesis reagents, are also useful for the preparation of activator.

The mechanism of phosphoramidite activation has
30 been studied (Vargeese, *supra*; Dahl et al., *Nucleic Acids Research*, 1987, 15, 1729-1743). The first step is the protonation of the trivalent phosphorus. The next step which is slower is the displacement of N,N-diisopropylamine by an activator such as 1-H tetrazole. Typically, in the

- 32 -

phosphitylation step of amidite based oligonucleotide synthesis, the 1-H tetrazole first participates as an acid, and then as a nucleophile.

Although not wanting to be bound by theory it is
5 thought that the mechanism of phosphitylation observed for the preferred pyridinium salts of the invention are not the same as when 1-H tetrazole is employed. As illustrated in the series of experiments of Example 12, the first step is seen as protonation of the phosphitylating reagent via
10 proton transfer from the activator. The second step is seen as the reaction of the activated phosphitylating species directly with the 3'-hydroxyl group of the nucleoside. Hence, the pyridine is not seen as a nucleophilic participant in the reaction scheme.

15 Pyridinium salts are non-explosive which make them substantially easier to store, use and dispose of relative to conventional activators such as 1H-tetrazole. Pyridinium salts and the starting materials necessary to generate them *in situ.*, are safely stored in large quantities. The
20 removal of pyridinium ion from reaction mixtures is easily performed by conversion to pyridine which is easily removed by evaporation. Furthermore, the cost of pyridinium salt is only \$0.10/gram compared to \$47.00/gram for 1H-tetrazole. This cost differential results in substantive cost-savings
25 for large scale manufacture of oligonucleotide drugs.

Another advantage that pyridinium salts have over conventional activators is their solubility in organic solvents. The solubility of pyridinium salts is significantly higher in solvents such as acetonitrile,
30 dichloromethane, and ethyl acetate than 1H-tetrazole. For example the solubility of pyridinium trifluoroacetate in acetonitrile is greater than 1 molar which is more than twice the solubility of 1H-tetrazole in acetonitrile which is about 0.5 molar under identical conditions. As a result of this increased
35 solubility the volume of solvents used during

phosphitylation can be greatly reduced. Another result of the increased solubility is that other solvent systems can be used giving enhanced results that are not feasible with activators such as 1H-tetrazole. The improved process of the present invention is performed using a solvent that can dissolve protected nucleosides. Preferred solvents include dichloromethane, dichloroethane, acetonitrile, tetrahydrofuran, ethyl acetate and mixtures thereof. In a preferred embodiment the improved process is performed using dichloromethane.

The use of pyridinium salts as activators improves the purity of the final phosphitylated material relative to conventional activators such as 1H-tetrazole. This improved purity results from a less acidic reaction medium when pyridinium salts are used. Pyridinium salts also provide a less acidic reaction environment than is observed when using more reactive phosphitylating reagents such as chloro-(2-cyanoethoxy)-N,N-diisopropylaminophosphine. This reduction in acidity leads to no loss of 5'-O-protection (see Example 9) which is always a problem with conventional more acidic activators. There is also no depurination seen (see Example 11) with the use of pyridinium activators. The fact that there are less undesired products as a result of depurination and deprotection simplifies purification of desired phosphoramidites.

A number of chemical functional groups present in the nucleosidic compounds of the invention can be protected and subsequently deblocked to the deprotected form. In general, a blocking group renders a chemical functionality of a molecule inert to specific reaction conditions and can later be removed from such functionality in a molecule without substantially damaging or altering the remainder of the molecule (Green and Wuts, Protective Groups in Organic Synthesis, 2d edition, John Wiley & Sons, New York, 1991). Common protecting groups that are routinely used during

oligonucleotide synthesis are disclosed in Agrawal, et al.,
Protocols for Oligonucleotide Conjugates, Eds, Humana Press;
New Jersey, 1994; Vol. 26 pp. 1-72.

Nucleosidic compounds according to the present
5 invention include monomeric and linked nucleosides. The
term "nucleoside" is intended to include naturally occurring
nucleosides and nucleosides having modified nucleobases
and/or modified sugar moieties. Internucleoside linkages
between linked nucleosides comprise native phosphodiester
10 linkages as well as modified linkages such as phosphoro-
thioate linkages. Other internucleoside linkages as is
known in the art are also amenable to the present invention.

As used in the present application the term
"nucleobase" is intended to include naturally occurring
15 nucleobases such as for example adenine, guanine, cytosine,
uridine, and thymine, as well as nucleobases that are
modified such as xanthine, hypoxanthine, 2-aminoadenine, 6-
methyl and other alkyl derivatives of adenine and guanine,
2-propyl and other alkyl derivatives of adenine and guanine,
20 5-halo uracil and cytosine, 6-aza uracil, cytosine and
thymine, 5-uracil (pseudo uracil), 4-thio uracil, 8-halo,
amino, thiol, thioalkyl, hydroxyl and other 8-substituted
adenines and guanines, 5-trifluoromethyl and other 5-
substituted uracils and cytosines, 7-methylguanine. Further
25 purines and pyrimidines include those disclosed in United
States Patent No. 3,687,808, those disclosed in the *Concise
Encyclopedia Of Polymer Science And Engineering*, pages 858-
859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, and
those disclosed by Englisch et al., *Angewandte Chemie*,
30 *International Edition* 1991, 30, 613, Limbach, A., et al.,
Nucleic Acids Research, 1994, 22, 2183-2196.

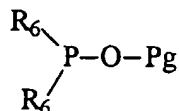
Sugar modifications are known in the prior art and
include for example 2' substituents such as F and 2'-O-
substituents such as substituted or unsubstituted C₁-C₁₀
35 alkyl, C₂-C₁₀ alkenyl, C₂-C₁₀ alkynyl, ethers and polyethers

- 35 -

wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto residues.

Modified internucleoside linkages are known in the prior art and include for example methylphosphonates, monothiophosphates, dithiophosphates, phosphoramidates, phosphate esters, bridged phosphoroamidates, bridged phosphorothioates, bridged methylenephosphonates, dephospho internucleotide analogs with siloxane bridges, carbonate bridges, carboxymethyl ester bridges, acetamide bridges, carbamate bridges, thioether, sulfoxy, sulfono bridges, various "plastic" DNAs, α -anomeric bridges, and borane derivatives.

Phosphitylating reagents that are amenable to the present invention require an activating agent prior to being susceptible to nucleophilic attack from an unprotected 2', 3' or 5' hydroxyl group. Included in this group are phosphitylating reagents having the formula below:



wherein the constituent variables are as defined above.

A more preferred group of phosphitylating reagents includes bis amidite reagent, bis(N,N-diisopropylamino)-2-methyltrifluoroacetyl aminoethoxyphosphine and bis(N,N-diisopropylamino)-2-diphenylmethylsilylethoxyphosphine, and bis(N,N-diisopropylamino)-2-(2'-acetoxyloxy)phenylethoxyphosphine.

In a preferred embodiment of the present invention pyridinium salts used as activators are selected to have Pka's of from about 5.2 to about 5.9. Preferred pyridinium salts in this group include pyridine hydrochloride,

pyridinium trifluoroacetate and pyridinium dichloroacetate.

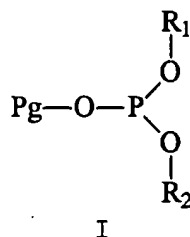
A summary of some phosphitylation activators, their optimal ratio, and exemplary choice of solvent is presented in Table 3 below. Several activators were studied
5 based on their pKa properties, steric bulk/size, cost, safety and scalability during manufacture of phosphoramidites. Also, the activator must act as an acid and have the capacity to transfer a proton to the phosphitylating reagent in an efficient manner. In
10 addition, when phosphitylating 5'-O-DMT-nucleosides the activator should be sufficiently mild to not cause destruction of the acid labile DMT protecting. Activators with pKa between 4.5 - 7 (see Figure 2) were chosen and studied herein. One preferred activator is the pyrimidine
15 derivative 2-amino-4,6-dimethylpyrimidine trifluoroacetate.

Some activators reported in the literature, for example 1-*H* tetrazole and diisopropylammonium tetrazolide, were judged unsuitable due to their high cost, safety in handling, explosive nature, and poor solubility in solvent
20 of choice. Preferred are those derived from pyridinium salts (pyridine hydrochloride, pyridinium trifluoroacetate, pyridinium triflate and pyridinium dichloroacetate) with a common pKa of 5.2. In further preferred embodiments, pyridinium hydrochloride and pyridinium triflate have been
25 shown to be particularly amenable to the methods of the invention, despite the hygroscopic nature of these salts. In particularly preferred embodiments, pyridinium trifluoroacetate activators are employed in the methods of the invention, because they possess an excellent safety
30 profile, low cost, and greater solubility in a range of solvents. Furthermore, the activator pyridinium trifluoroacetate was used to phosphitylate a variety of nucleoside derivatives (1-4, Figure 1) to provide excellent yields. In further preferred embodiments, pyridinium
35 dichloroacetate (see Example 8) also is useful as an

- 37 -

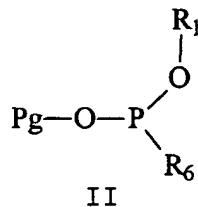
activator in the methods of the invention, and may have an advantage over pyridinium trifluoroacetate because pyridine and dichloroacetic acid is also used as a deblocking solution during oligonucleotide manufacturing, thus avoiding the storage and handling of an addition reagent.

In a further aspect, the present invention provides novel methods for the preparation of covalent intersugar linkages. In some preferred embodiments, the current invention presents methods for the preparation of a compound of Formula I:



wherein:

- R_1 is a nucleoside or an oligonucleotide;
- R_2 is a nucleoside linked to a solid support, or an oligonucleotide linked to a solid support;
- Pg is a phosphorus protecting group;
- comprising:
- providing a phosphoramidite of Formula II:



wherein R_6 is $-\text{N}(\text{R}_7)_2$ wherein R_7 is alkyl having from one to about six carbons; or R_7 is a heterocycloalkyl or heterocycloalkenyl ring containing from 4 to 7 atoms, and having up to 3 heteroatoms selected from nitrogen, sulfur, and oxygen;

- 38 -

and reacting said phosphoramidite with a hydroxyl group of a nucleoside linked to a solid support, or an oligonucleotide linked to a solid support;

said reaction being performed in the presence of
5 an activating reagent, said activating reagent comprising at least one pyridinium salt and at least one substituted imidazole.

The methods of the invention are applicable to the preparation of intersugar linkages including those
10 represented by Formula I above. According to some preferred embodiments of the methods of the invention, a protected phosphoramidite having Formula II is reacted with a hydroxyl group of a sugar moiety of a nucleoside or oligonucleotide. In more preferred embodiments, the nucleoside or
15 oligonucleotide are linked to a solid support, as in, for example, standard solid phase oligonucleotide synthetic regimes.

In the methods of the invention, the reaction of the phosphoramidite and the hydroxyl group is performed in
20 the presence of an activating reagent. As used herein, the term "activating reagent" is intended to mean a reagent that, at a minimum, includes at least one pyridinium salt. It is preferred that the activating reagent also contain at least one imidazole or substituted imidazole, in addition to
25 the pyridinium salt.

The reaction of the phosphoramidite and the hydroxyl group in the presence of the activating reagent can be performed in a solvent, such as acetonitrile.

Also provided in accordance with the present
30 invention are methods for the preparation of an oligonucleotide comprising the steps of:

providing a 3'-mononucleoside phosphoramidite or 3'-oligonucleotide phosphoramidite; and

reacting said 3'-mononucleoside phosphoramidite or
35 3'-oligonucleotide phosphoramidite with the 5'-hydroxyl of a

nucleoside, nucleotide, or oligonucleotide in the presence of an activating reagent;

said activating reagent comprising at least one pyridinium salt and at least one substituted imidazole.

5 In some preferred embodiments, the 3'-mononucleoside phosphoramidite or oligonucleotide phosphoramidite is reacted with the 5'-hydroxyl of a solid-support bound nucleoside, nucleotide or oligonucleotide.

10 In further preferred embodiments of the methods of the invention, the oligonucleotide comprises phosphorothioate intersugar linkages.

The present invention also provides synthetic methods comprising:

providing a phosphoramidite of formula:

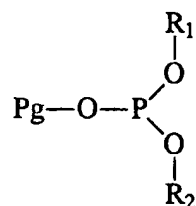


wherein:

R_6 is morpholino or dialkylamino;

Pg is a phosphorus protecting group;

20 and reacting said phosphoramidite with a hydroxyl group of a nucleoside linked to a solid support, or an oligonucleotide linked to a solid support, to form a compound of formula:



wherein:

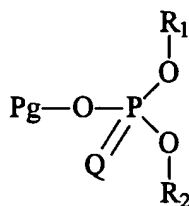
25 R_1 is a mononucleoside or an oligonucleotide;

- 40 -

R_2 is a nucleoside linked to a solid support, or an oligonucleotide linked to a solid support;

said reaction being performed in the presence of an activating reagent, said activating reagent comprising at least one pyridinium salt and at least one substituted imidazole; and

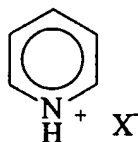
oxidizing or sulfurizing said compound to form a compound of formula:



wherein Q is O or S.

In some preferred embodiments of the forgoing methods, the substituted imidazole is 1-methylimidazole.

In further preferred embodiments, the pyridinium salt has the formula

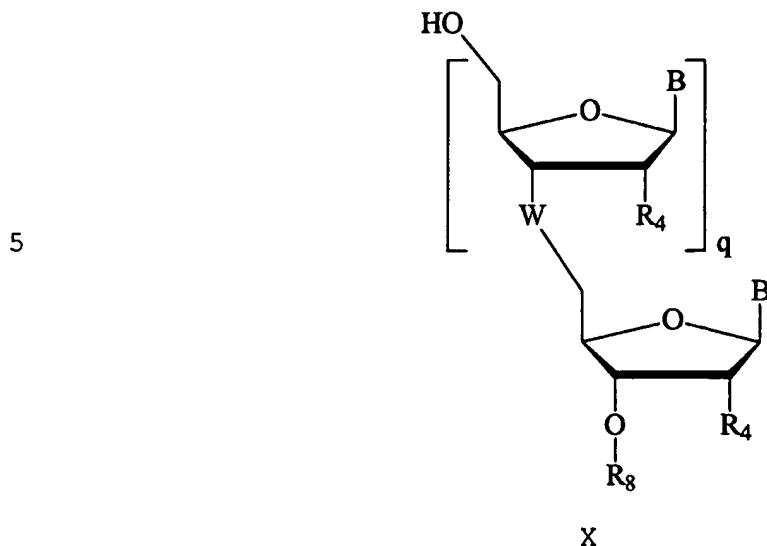


where X^- is an anion such as, for example, trifluoroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br , ^-O -trifluorosulfonyl, hexafluorophosphate, or tetrafluoroborate, with trifluoroacetate being preferred.

In some preferred embodiments, the compound is a single nucleoside or a nucleoside that is part of a larger molecule such as an oligonucleotide or an oligonucleotide analog. The improved process of the present invention offers significant advantages over traditionally used processes.

In a further aspect of the invention, synthetic methods are provided comprising:

providing a compound of Formula X:



wherein:

B is a nucleobase;

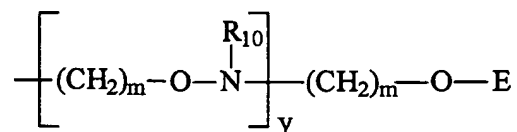
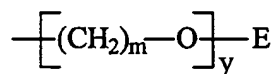
10 R₈ is H, a hydroxyl protecting group, or a linker connected to a solid support;

W is an optionally protected internucleoside linkage;

q is 0 to about 50;

R₄ is H, F, O-R, S-R or N-R(R₁₀);

15 R is H, a protecting group, or has one of the formulas:



where

each m is independently from 1 to 10;

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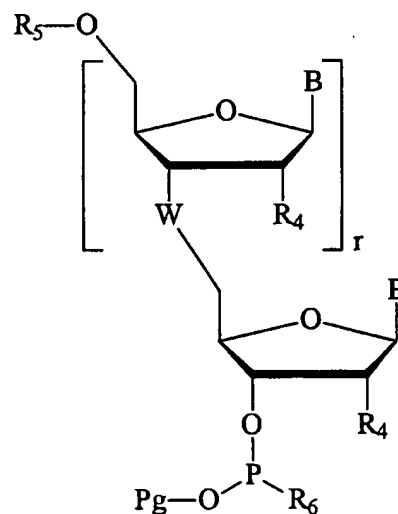
y is from 0 to 10;

E is H, a hydroxyl protecting group, C_1-C_{10} alkyl, $N(R_{10})(R_{11})$ or $N=C(R_{10})(R_{11})$; substituted or unsubstituted C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto residues; and

each R_{10} or R_{11} is, independently, H, substituted or unsubstituted C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto residues; alkylthioalkyl, a nitrogen protecting group, or R_{10} and R_{11} , together, are a nitrogen protecting group or wherein R_{10} and R_{11} are joined in a ring structure that can include at least one heteroatom selected from N and O;

or R is $-CH_2-CH_2-O-CH_2-CH_2-N(R_{10})(R_{11})$;

reacting the compound of Formula X in the presence of an activating reagent with a compound of Formula XI:



XI

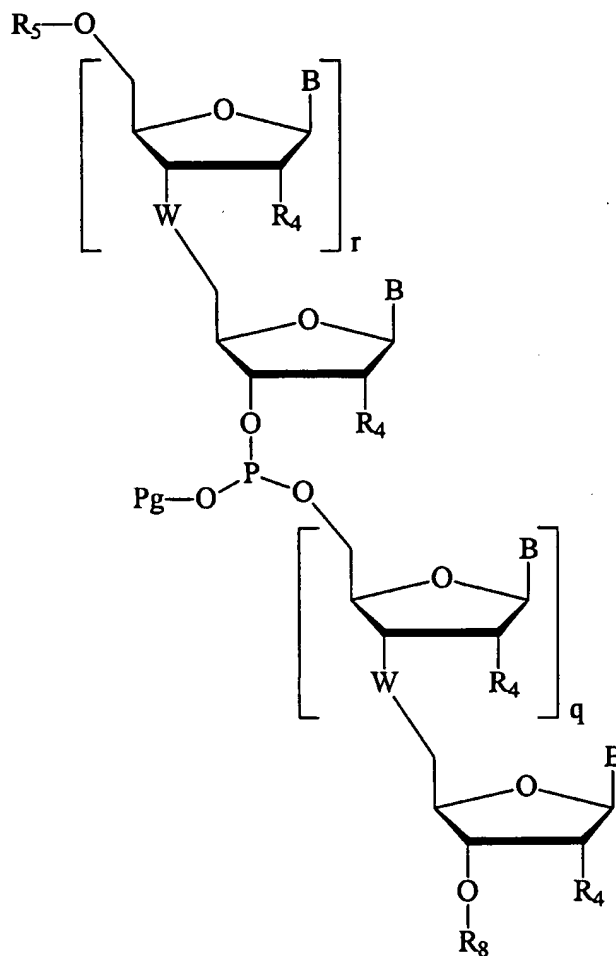
where r is 0 to about 50;

R_5 is a hydroxyl protecting group;

R_6 is $-N(R_7)_2$ wherein R_7 is alkyl having from one to

about six carbons; or R_7 is a heterocycloalkyl or heterocycloalkenyl ring containing from 4 to 7 atoms, and having up to 3 heteroatoms selected from nitrogen, sulfur, and oxygen;

5 to form a compound of Formula XII:



XII

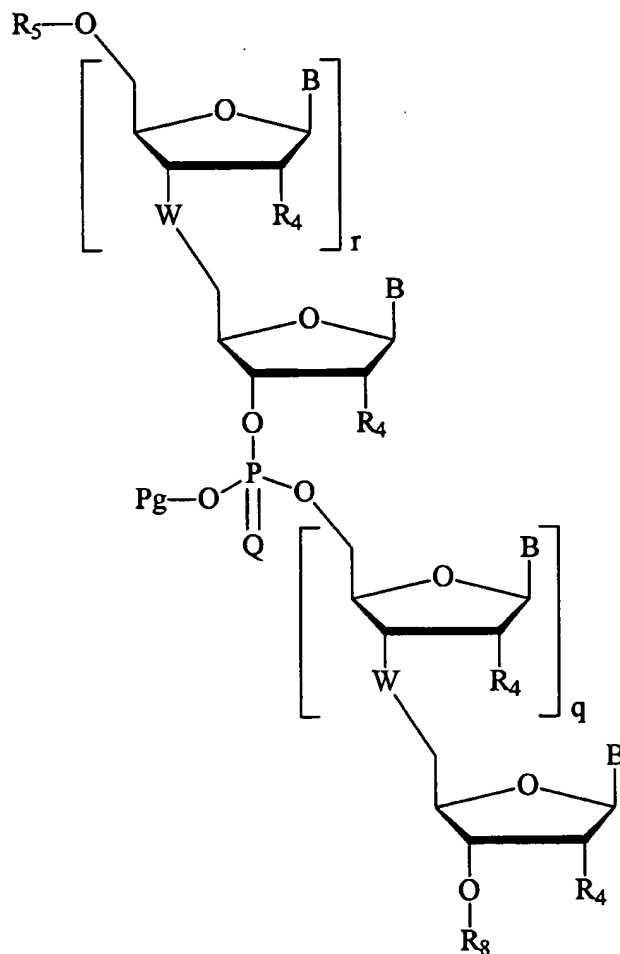
wherein said activating reagent comprises at least one pyridinium salt and one substituted imidazole.

10 Preferably, the activator has the formula G^+U^- , where G^+ is selected from the group consisting of pyridinium, imidazolium, and benzimidazolium; and U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate,

triflate, hydrochloride, trifluoroacetate, dichloroacetate, O-mesyl , O-tosyl , Br , and $\text{O-trifluorosulfonyl}$.

Preferably, the compound of Formula XII can then be oxidized or sulfurized to form a compound of Formula

5 XIII:



XIII

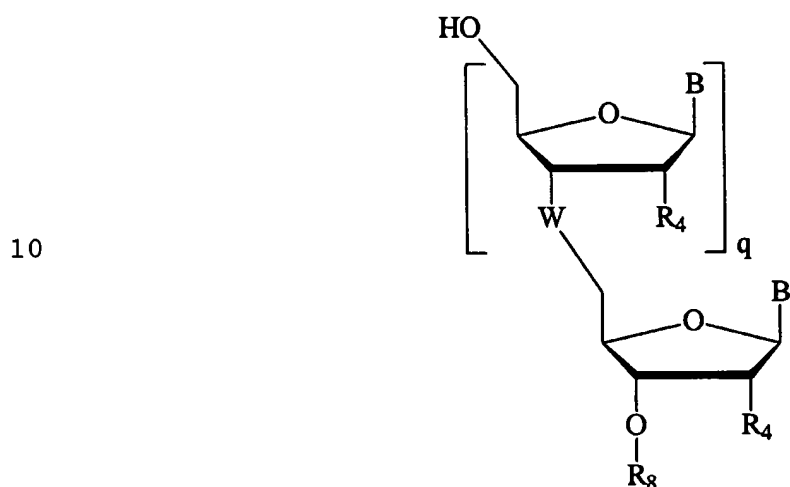
where Q is O or S.

After completion of synthetic regime, the final
10 product is then cleaved from the solid support to produce a further compound of Formula X.

In a further aspect of the invention, methods are provided for the preparation of internucleoside linkages between nucleosides having 2'-substituents, using an
15 imidazolium triflate activating reagent. As used herein,

the term "an imidazolium triflate" denotes imidazolium triflate, as well as substituted imidazolium triflates wherein the substituents are one or more electron withdrawing groups such as, for example, halogen, nitro or cyano. Preferably, the activating reagent is unsubstituted imidazolium triflate. In some preferred embodiments, these methods comprise:

providing a compound of Formula X:



X

wherein:

B is a nucleobase;

R₈ is H, a hydroxyl protecting group, or a linker
15 connected to a solid support;

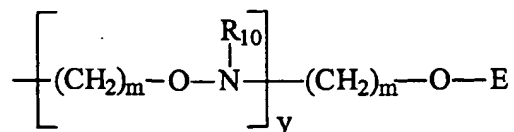
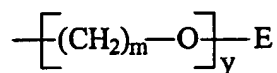
W is an optionally protected internucleoside linkage;

q is 0 to about 50;

R_4 is H, F, O-R, S-R or N-R (R_{10});

20 R is H, a protecting group, or has one of the
formulas:

- 46 -



where

each m is independently from 1 to 10;

y is from 0 to 10;

5 E is H, a hydroxyl protecting group, C₁-C₁₀ alkyl, N(R₁₀) (R₁₁) or N=C(R₁₀) (R₁₁); substituted or unsubstituted C₁-C₁₀ alkyl, C₂-C₁₀ alkenyl, C₂-C₁₀ alkynyl, wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto
10 residues; and

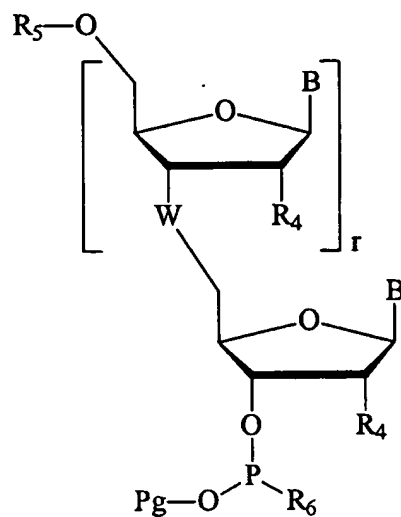
each R₁₀ or R₁₁ is, independently, H, substituted or unsubstituted C₁-C₁₀ alkyl, C₂-C₁₀ alkenyl, C₂-C₁₀ alkynyl, wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto
15 residues; alkylthioalkyl, a nitrogen protecting group, or R₁₀ and R₁₁, together, are a nitrogen protecting group or wherein R₁₀ and R₁₁ are joined in a ring structure that can include at least one heteroatom selected from N and O;

or R is -CH₂-CH₂-O-CH₂-CH₂-N(R₁₀) (R₁₁);

20 provided that R₁₄ is not H or OH;

reacting the compound of Formula X in the presence of an activator with a compound of Formula XI:

- 47 -

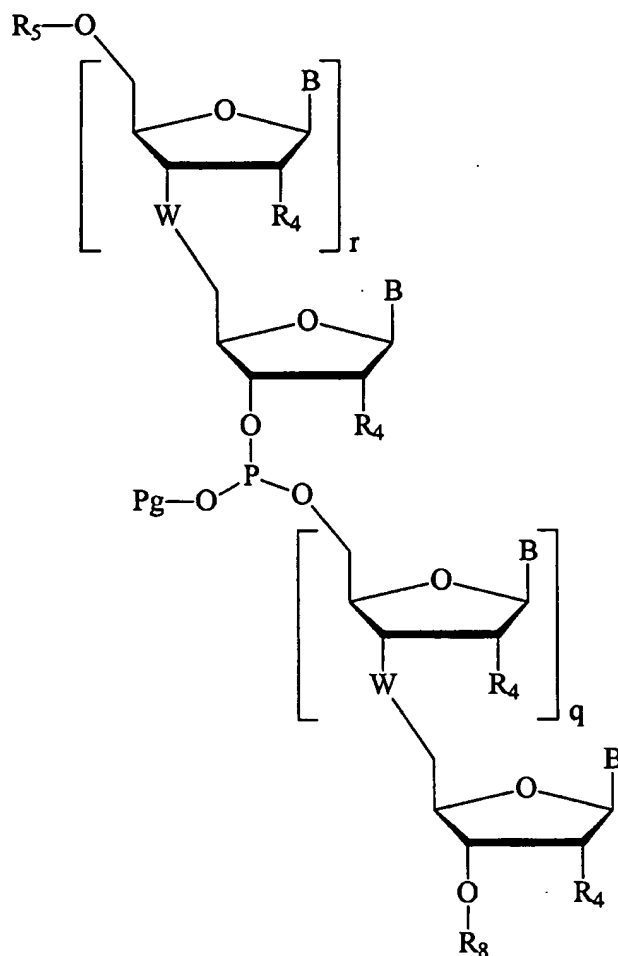


XI

where r is 0 to about 50;

5 R_5 is a hydroxyl protecting group;

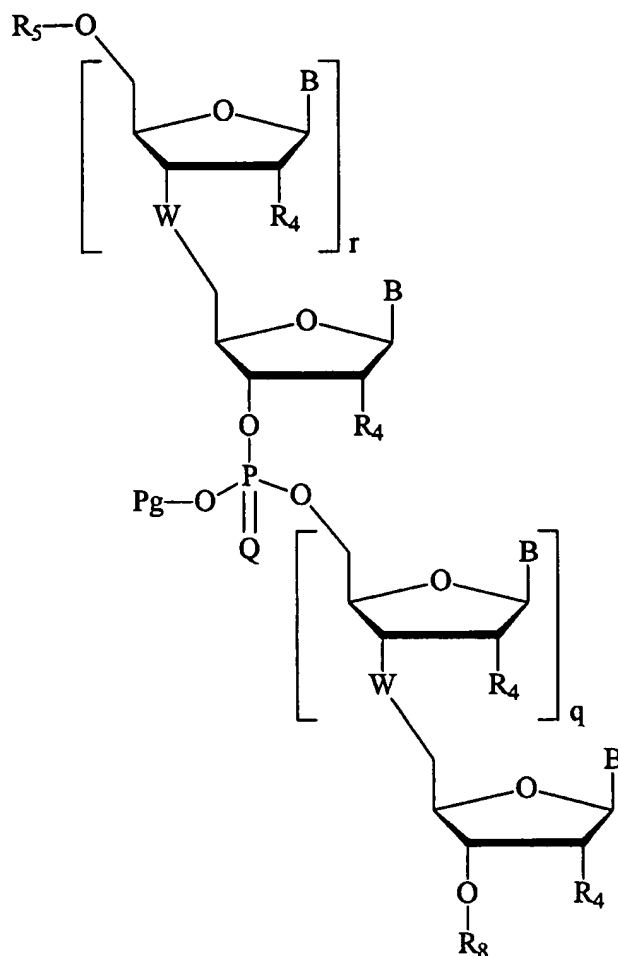
R_6 is $-N(R_7)_2$ wherein R_7 is alkyl having from one to about six carbons; or R_7 is a heterocycloalkyl or heterocycloalkenyl ring containing from 4 to 7 atoms, and having up to 3 heteroatoms selected from nitrogen, sulfur,
 10 and oxygen;
 to form a compound of Formula XII:



XII

wherein the activator has the formula G^+U^- ,
 where G^+ is selected from the group consisting of pyridinium,
 5 imidazolium, and benzimidazolium; and U^- is selected from the
 group consisting of hexafluorophosphate, tetrafluoroborate,
 triflate, hydrochloride, trifluoroacetate, dichloroacetate,
 ^-O -mesyl, ^-O -tosyl, ^-Br , and ^-O -trifluorosulfonyl.
 Preferably, the activator is imidazolium triflate.

10 Some further preferred embodiments further
 comprise oxidizing or sulfurizing the compound of Formula
 XII to form a compound of Formula XIII:



XIII

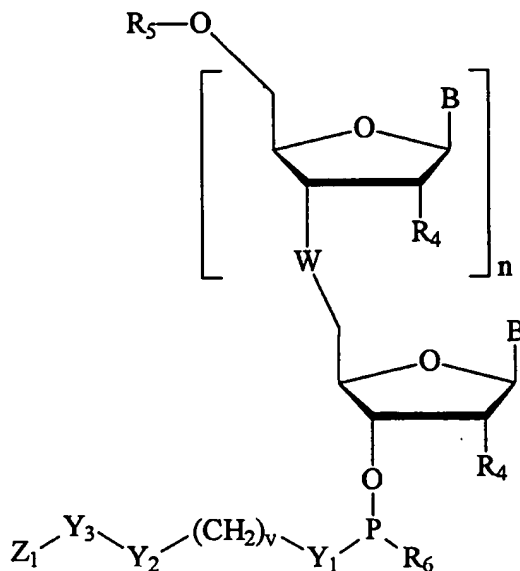
where Q is O or S.

Some further preferred embodiments of the methods
 5 further comprising a capping step, which is preferably
 performed prior to oxidation.

Some further preferred embodiments further
 comprising the step of cleaving the oligomeric compound to
 produce a further compound of formula X.

10 In a further aspect of the invention, synthetic
 methods are provided for the preparation of dimeric and
 higher order oligonucleotides having at least one
 bioreversible protecting group that confers enhanced
 chemical and biophysical properties. In some preferred
 15 embodiments, these methods comprise:
 providing a compound of Formula XX:

- 50 -

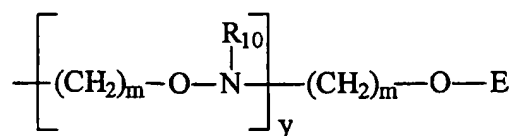
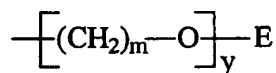


XX

wherein:

 R_4 is H, F, O-R, S-R or N-R(R_{10});

5 R is H, a protecting group, or has one of the
formulas:



where

each m is independently from 1 to 10;

10 y is from 0 to 10;

E is H, a hydroxyl protecting group, $\text{C}_1\text{--C}_{10}$ alkyl, N(R_{10}) (R_{11}) or $\text{N}=\text{C}(\text{R}_{10})$ (R_{11}); substituted or unsubstituted $\text{C}_1\text{--C}_{10}$ alkyl, $\text{C}_2\text{--C}_{10}$ alkenyl, $\text{C}_2\text{--C}_{10}$ alkynyl, wherein the substitutions are selected from one or several
15 halogen, cyano, carboxy, hydroxy, nitro and mercapto residues; and

- 51 -

each R_{10} or R_{11} is, independently, H, substituted or unsubstituted C_1 - C_{10} alkyl, C_2 - C_{10} alkenyl, C_2 - C_{10} alkynyl, wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto
5 residues; alkylthioalkyl, a nitrogen protecting group, or R_{10} and R_{11} , together, are a nitrogen protecting group or wherein R_{10} and R_{11} are joined in a ring structure that can include at least one heteroatom selected from N and O;

or R is $-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}_2-\text{N}(\text{R}_{10})(\text{R}_{11})$;

10 R_5 is a hydroxyl protecting group;

Z_1 is aryl having 6 to about 14 carbon atoms or alkyl having from one to about six carbon atoms;

Y_1 is O or S;

Y_2 is O or S;

15 Y_3 is $\text{C}(=\text{O})$ or S;

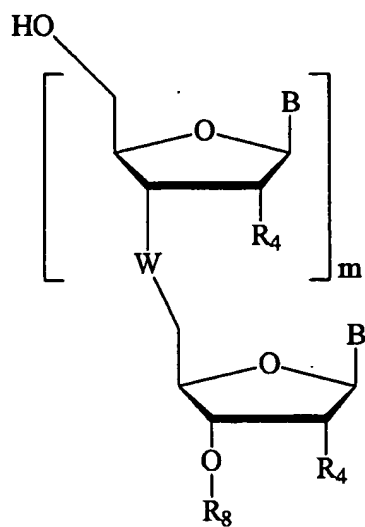
v is 2 to about 4;

B is a nucleobase;

R_6 is $-\text{N}(\text{R}_7)_2$ wherein R_7 is alkyl having from one to about six carbons; or R_7 is a heterocycloalkyl or
20 heterocycloalkenyl ring containing from 4 to 7 atoms, and having up to 3 heteroatoms selected from nitrogen, sulfur, and oxygen;

reacting said compound of Formula XX with a compound of Formula XXI:

- 52 -

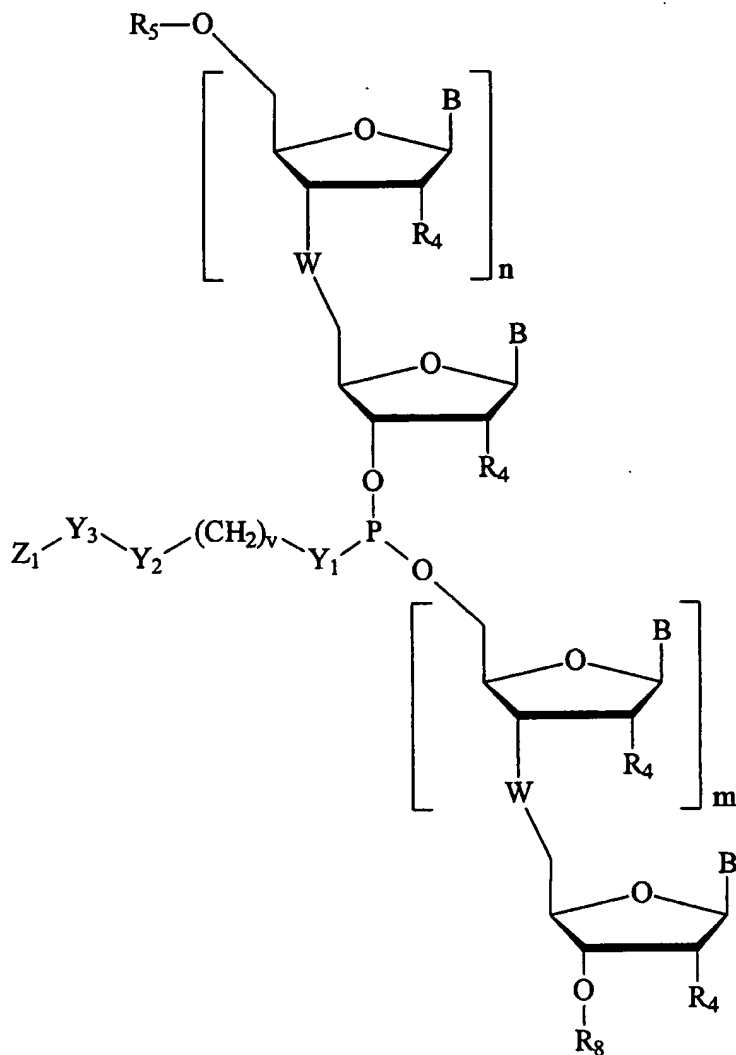


XXI

wherein:

- R_8 is H, a hydroxyl protecting group, or a linker
5 connected to a solid support;
in the presence of an activator to form a compound
of Formula XXII:

- 53 -



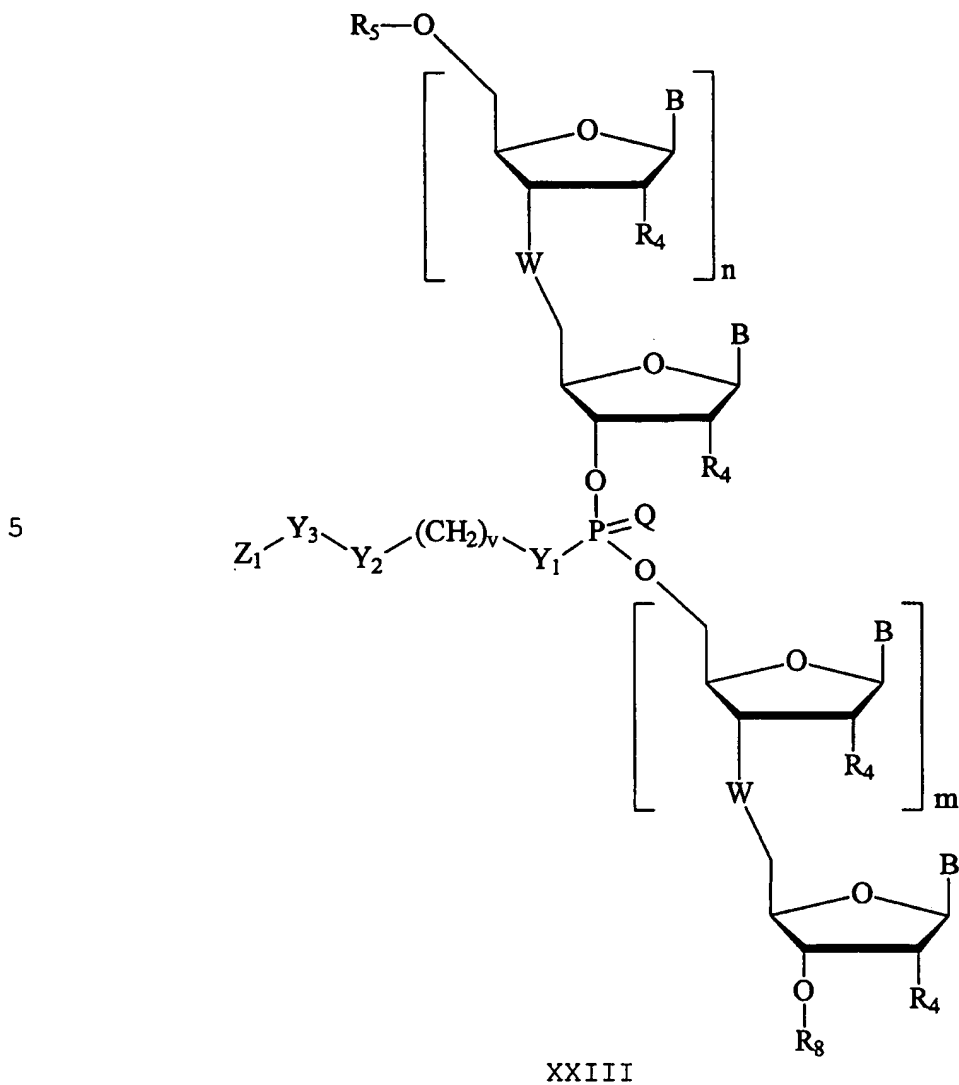
XXII

wherein said activator has the formula G^+U^- , where G^+ is selected from the group consisting of pyridinium, imidazolium, and benzimidazolium; and U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate, triflate, hydrochloride, trifluoroacetate, dichloroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br , and ^-O -trifluorosulfonyl. Preferably, the activator is an imidazolium triflate

10 activator.

Some preferred embodiments of the foregoing

methods further comprise oxidizing or sulfurizing the compounds of Formula XXII to form a compound of Formula XXIII:



where Q is O or S.

Some further preferred embodiments of the methods further comprising a capping step, which is preferably performed prior to oxidation.

10

Some further preferred embodiments further comprising the step of cleaving the oligomeric compound to produce a further compound of formula XXI.

Methods for the preparation of compound XX can be
5 found in copending application ser. nos. 09/066,638 and
09/095,822 filed April 24, 1998 and June 11, 1998,
respectively, which are assigned to the assignee of the
present application. The contents of the foregoing patent
applications are hereby incorporated by reference in their
10 entirety.

In further preferred embodiments, each of the
foregoing methods, are performed iteratively to produce an
oligonucleotide or analog thereof having a preselected
nucleotide base sequence. In general, the phosphorus
15 protecting groups, designated "Pg" in the formulas herein,
are removed at the end of the synthetic regime, preferably
at the time that the completed oligonucleotide or analog is
cleaved from the solid support. However, in some preferred
embodiments, the methods of the invention are beneficially
20 employed to provide oligonucleotide analogs having at least
one bioreversible protecting group that confers enhanced
chemical and biophysical properties. See copending
applications ser. nos. 09/066,638 and 09/095,822 filed April
24, 1998 and June 11, 1998, respectively. The
25 bioreversible protecting groups further lend nuclease
resistance to the oligonucleotides. The bioreversible
protecting groups are removed in a cell, in the cell
cytosol, or *in vitro* in cytosol extract, by endogenous
enzymes. In certain preferred oligonucleotides of the
30 invention the bioreversible protecting groups are designed
for cleavage by carboxyesterases to yield unprotected
oligonucleotides.

Preferably, the bioreversible protecting group has
the Formula $Z_1-Y_3-Y_2-(CH_2)_v-Y_1-$, wherein the constituent
35 variable are as defined above. In some preferred

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embodiments, Y_1 and Y_2 are each O, Y_3 is S, and Z is methyl or t-butyl, with t-butyl being preferred.

One particular advantage of the present invention is that the assembly of oligonucleotides and analogs thereof
5 containing the bioreversible protecting group in accordance with the methods of the invention does not require protection for exocyclic nucleobase amino moieties, thus conferring significant benefit in expense, effort, and yield.

10 In preferred embodiments, the methods of the invention are used for the preparation of oligonucleotides and their analogs. As used herein, the term "oligonucleotide" is intended to include both naturally occurring and non-naturally occurring (i.e., "synthetic")
15 oligonucleotides. Naturally occurring oligonucleotides are those which occur in nature; for example ribose and deoxyribose phosphodiester oligonucleotides having adenine, guanine, cytosine, thymine and uracil nucleobases. As used herein, non-naturally occurring oligonucleotides are
20 oligonucleotides that contain modified sugar, internucleoside linkage and/or nucleobase moieties. Such oligonucleotide analogs are typically structurally distinguishable from, yet functionally interchangeable with, naturally occurring or synthetic wild type oligonucleotides.
25 Thus, non-naturally occurring oligonucleotides include all such structures which function effectively to mimic the structure and/or function of a desired RNA or DNA strand, for example, by hybridizing to a target.

Representative nucleobases include adenine,
30 guanine, cytosine, uridine, and thymine, as well as other non-naturally occurring and natural nucleobases such as xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 5-halo uracil and
35 cytosine, 6-azo uracil, cytosine and thymine, 5-uracil

(pseudo uracil), 4-thiouracil, 8-halo, oxa, amino, thiol, thioalkyl, hydroxyl and other 8-substituted adenines and guanines, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine. Further naturally and non
5 naturally occurring nucleobases include those disclosed in U.S. Patent No. 3,687,808 (Merigan, et al.), in chapter 15 by Sanghvi, in *Antisense Research and Application*, Ed. S. T. Crooke and B. Lebleu, CRC Press, 1993, in Englisch et al., *Angewandte Chemie*, International Edition, 1991, 30, 613-722
10 (see especially pages 622 and 623, and in the *Concise Encyclopedia of Polymer Science and Engineering*, J.I. Kroschwitz Ed., John Wiley & Sons, 1990, pages 858-859, Cook, *Anti-Cancer Drug Design* 1991, 6, 585-607, each of which are hereby incorporated by reference in their
15 entirety). The term "nucleosidic base" is further intended to include heterocyclic compounds that can serve as like nucleosidic bases including certain "universal bases" that are not nucleosidic bases in the most classical sense but serve as nucleosidic bases. Especially mentioned as a
20 universal base is 3-nitropyrrole.

Representative 2' sugar modifications (moiety R₁ in the formulas described herein) amenable to the present invention include fluoro, O-alkyl, O-alkylamino, O-alkylalkoxy, protected O-alkylamino, O-alkylaminoalkyl, O-
25 alkyl imidazole, and polyethers of the formula (O-alkyl)_m, where m is 1 to about 10. Preferred among these polyethers are linear and cyclic polyethylene glycols (PEGs), and (PEG)-containing groups, such as crown ethers and those which are disclosed by Ouchi, et al., *Drug Design and*
30 *Discovery* 1992, 9, 93, Ravasio, et al., *J. Org. Chem.* 1991, 56, 4329, and Delgado et. al., *Critical Reviews in Therapeutic Drug Carrier Systems* 1992, 9, 249, each of which are hereby incorporated by reference in their entirety. Further sugar modifications are disclosed in Cook, P.D.,

supra. Fluoro, O-alkyl, O-alkylamino, O-alkyl imidazole, O-alkylaminoalkyl, and alkyl amino substitution is described in United States Patent Application serial number 08/398,901, filed March 6, 1995, entitled Oligomeric
5 Compounds having Pyrimidine Nucleotide(s) with 2' and 5' Substitutions, hereby incorporated by reference in its entirety.

Sugars having O-substitutions on the ribosyl ring are also amenable to the present invention. Representative
10 substitutions for ring O include S, CH₂, CHF, and CF₂, see, e.g., Secrist, et al., *Abstract 21, Program & Abstracts, Tenth International Roundtable, Nucleosides, Nucleotides and their Biological Applications*, Park City, Utah, Sept. 16-20, 1992, hereby incorporated by reference in its entirety.

15 As used herein, the term "alkyl" includes but is not limited to straight chain, branch chain, and alicyclic hydrocarbon groups. Alkyl groups of the present invention may be substituted. Representative alkyl substituents are disclosed in United States Patent No. 5,212,295, at column
20 12, lines 41-50, hereby incorporated by reference in its entirety.

"Aryl" groups are aromatic cyclic compounds including but not limited to phenyl, naphthyl, anthracyl, phenanthryl, pyrenyl, and xylyl.

25 In general, the term "hetero" denotes an atom other than carbon, preferably but not exclusively N, O, or S. Accordingly, the term "heterocycloalkyl" denotes an alkyl ring system having one or more heteroatoms (i.e., non-carbon atoms). Preferred heterocycloalkyl groups include,
30 for example, morpholino groups. As used herein, the term "heterocycloalkenyl" denotes a ring system having one or more double bonds, and one or more heteroatoms. Preferred heterocycloalkenyl groups include, for example, pyrrolidino groups.

35 In some preferred embodiments of the invention R₈

can be a linker connected to a solid support. Solid supports are substrates which are capable of serving as the solid phase in solid phase synthetic methodologies, such as those described in Caruthers U.S. Patents Nos. 4,415,732; 4,458,066; 4,500,707; 4,668,777; 4,973,679; and 5,132,418; and Koster U.S. Patents Nos. 4,725,677 and Re. 34,069.

Linkers are known in the art as short molecules which serve to connect a solid support to functional groups (e.g., hydroxyl groups) of initial synthon molecules in solid phase synthetic techniques. Suitable linkers are disclosed in, for example, *Oligonucleotides And Analogues A Practical Approach*, Ekstein, F. Ed., IRL Press, N.Y, 1991, Chapter 1, pages 1-23, hereby incorporated by reference in its entirety.

Preferred linkers for use in linking the growing oligonucleotide chain to the solid support in some preferred embodiments of the methods of the invention will be cleaved by reagents that do not result in removal of the $-Y_1-(CH_2)_q-Y_2-Y_3-Z$ protecting group. One such linker is the oxalyl linker (Alul, R.H., et al., *Nucl. Acids Res.* 1991, 19, 1527) between a LCAA-CPG solid support and the oligomer. Other photolabile supports have been reported (Holmes et al., *J. Org. Chem.* 1997, 62, 2370-2380; Greenberg et al., *Tetrahedron Lett.* 1993, 34, 251-254). The o-nitrobenzyl functionalized solid support has been previously reported (Dell'Aquila et al., *Tetrahedron Lett.* 1997, 38, 5289-5292). Another preferred method of cleavage without removal of internucleoside protecting groups is by irradiation with ultraviolet light in aqueous acetonitrile.

Solid supports according to the invention include those generally known in the art to be suitable for use in solid phase methodologies, including, for example, controlled pore glass (CPG), oxalyl-controlled pore glass (see, e.g., Alul, et al., *Nucleic Acids Research* 1991, 19,

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1527, hereby incorporated by reference in its entirety),
TentaGel Support, an aminopolyethyleneglycol derivatized
support (see, e.g., Wright, et al., *Tetrahedron Letters*
1993, 34, 3373, hereby incorporated by reference in its
5 entirety) and Poros, a copolymer of
polystyrene/divinylbenzene.

In some preferred embodiments of the invention R_5
or R_8 can be a hydroxyl protecting group. A wide variety of
hydroxyl protecting groups can be employed in the methods of
10 the invention. Preferably, the protecting group is stable
under basic conditions but can be removed under acidic
conditions. In general, protecting groups render chemical
functionalities inert to specific reaction conditions, and
can be appended to and removed from such functionalities in
15 a molecule without substantially damaging the remainder of
the molecule. Representative hydroxyl protecting groups are
disclosed by Beaucage, et al., *Tetrahedron* 1992, 48, 2223-
2311, and also in Greene and Wuts, *Protective Groups in*
Organic Synthesis, Chapter 2, 2d ed, John Wiley & Sons, New
20 York, 1991, each of which are hereby incorporated by
reference in their entirety. Preferred protecting groups
used for R_5 and R_8 include dimethoxytrityl (DMT),
monomethoxytrityl, 9-phenylxanthen-9-yl (Pixyl) and 9-(p-
methoxyphenyl)xanthen-9-yl (Mox). The R_5 or R_8 group can be
25 removed from oligomeric compounds of the invention by
techniques well known in the art to form the free hydroxyl.
For example, dimethoxytrityl protecting groups can be
removed by protic acids such as formic acid, dichloroacetic
acid, trichloroacetic acid, p-toluene sulphonic acid or with
30 Lewis acids such as for example zinc bromide. See for
example, Greene and Wuts, *supra*.

In some preferred embodiments of the invention
amino groups are appended to alkyl or other groups, such as,
for example, 2'-alkoxy groups (e.g., where R_1 is alkoxy).
35 Such amino groups are also commonly present in naturally

occurring and non-naturally occurring nucleobases. It is generally preferred that these amino groups be in protected form during the synthesis of oligomeric compounds of the invention. Representative amino protecting groups suitable
5 for these purposes are discussed in Greene and Wuts, *Protective Groups in Organic Synthesis*, Chapter 7, 2d ed, John Wiley & Sons, New York, 1991. Generally, as used herein, the term "protected" when used in connection with a molecular moiety such as "nucleobase" indicates that the
10 molecular moiety contains one or more functionalities protected by protecting groups.

Sulfurizing agents used during oxidation to form phosphorothioate and phosphorodithioate linkages include Beaucage reagent (see e.g. Iyer, et.al., *J. Chem. Soc.* **1990**,
15 112, 1253-1254, and Iyer, et.al., *J. Org. Chem.* **1990**, 55, 4693-4699); tetraethylthiuram disulfide (see e.g., Vu, et al., *Tetrahedron Lett.* **1991**, 32, 3005-3008); dibenzoyl tetrasulfide (see e.g., Rao, et.al., *Tetrahedron Lett.* **1992**, 33, 4839-4842); di(phenylacetyl)disulfide (see e.g., Kamer,
20 *Tetrahedron Lett.* **1989**, 30, 6757-6760); Bis(O,O-diisopropoxy phosphinothioyl)disulfide (see Stec et al., *Tetrahedron Lett.* **1993**, 34, 5317-5320); 3-ethoxy-1,2,4-dithiazoline-5-one (see *Nucleic Acids Research*, **1996** 24, 1602-1607, and *Nucleic Acids Research*, **1996** 24, 3643-3644); Bis(p-
25 chlorobenzenesulfonyl)disulfide (see *Nucleic Acids Research*, **1995** 23, 4029-4033); sulfur, sulfur in combination with ligands like triaryl, trialkyl, triaralkyl, or trialkaryl phosphines. The foregoing references are hereby incorporated by reference in their entirety.

30 Useful oxidizing agents used to form the phosphodiester or phosphorothioate linkages include iodine/tetrahydrofuran/ water/pyridine or hydrogen peroxide/water or tert-butyl hydroperoxide or any peracid like m-chloroperbenzoic acid. In the case of sulfurization

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the reaction is performed under anhydrous conditions with the exclusion of air, in particular oxygen whereas in the case of oxidation the reaction can be performed under aqueous conditions.

5 Oligonucleotides or oligonucleotide analogs according to the present invention hybridizable to a specific target preferably comprise from about 5 to about 50 monomer subunits. It is more preferred that such compounds
10 comprise from about 10 to about 30 monomer subunits, with 15 to 25 monomer subunits being particularly preferred. When used as "building blocks" in assembling larger oligomeric compounds (*i.e.*, as synthons of Formula II), smaller oligomeric compounds are preferred. Libraries of dimeric, trimeric, or higher order compounds of general Formula II
15 can be prepared for use as synthons in the methods of the invention. The use of small sequences synthesized via solution phase chemistries in automated synthesis of larger oligonucleotides enhances the coupling efficiency and the purity of the final oligonucleotides. See for example:
20 Miura, *et al.*, *Chem. Pharm. Bull.* **1987**, *35*, 833-836; Kumar, *et al.*, *J. Org. Chem.* **1984**, *49*, 4905-4912; Bannwarth, *Helvetica Chimica Acta* **1985**, *68*, 1907-1913; Wolter, *et al.*, *Nucleosides and Nucleotides* **1986**, *5*, 65-77, each of which are hereby incorporated by reference in their entirety.

25 The oligonucleotides produced by preferred embodiments of the methods of the invention having bioreversible protecting groups are also referred to in this specification as pro-oligonucleotides. Such pro-oligonucleotides are capable of improved cellular lipid
30 bilayers penetrating potential as well as resistance to exo- and endonuclease degradation *in vivo*. In cells, the bioreversible protecting groups are removed in the cell cytosol by endogenous carboxyesterases to yield biologically active oligonucleotide compounds that are capable of
35 hybridizing to and/or having an affinity for specific

nucleic acid.

Additional advantages and novel features of this invention will become apparent to those skilled in the art upon examination of the examples thereof provided below, which should not be construed as limiting the appended claims.

Preparation of Phosphoramidites

Example 1

General phosphitylation procedure using 2'-deoxy-5'-O-DMT nucleosides with pyridinium trifluoroacetate

To a sample of 2'-deoxy-5'-O-DMT-nucleoside (2'-O-deoxy-5'-O-DMT-6-N-benzoyladenosine, 2'-O-deoxy-5'-O-DMT-4-N-benzoylcytidine, 2'-O-deoxy-5'-O-DMT-2-N-isobutyrylguanosine and 2'-O-deoxy-5'-O-DMT-thymidine) (1a-1d, Figure 1, 10 mmol, 5.45 - 6.40 g) in dry dichloromethane (25 mL) was added bisamidite reagent (2-cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite, 5, figure 1, 3.81 mL, 3.62 g, 12 mmol) at ambient temperature under argon. Pyridinium trifluoroacetate (2.32 g, 12 mmol) was added and the reaction mixture was stirred at ambient temperature for 2-3 hours. The reaction was diluted with dichloromethane (35 mL), and washed with of saturated $\text{NaHCO}_3/\text{H}_2\text{O}$ (30 mL). The organic layer was separated, dried (Na_2SO_4), evaporated, and purified on a short silica gel column. The amidite product was eluted with 60-80% EtOAc/hexanes (1% triethylamine) with the exact concentration dependent to the respective amidite being purified. The appropriate fractions were collected and evaporated to give the respective amidite product (6a-d, Figure 1) as a white foam in ~ 80% yield.

Example 2

General phosphitylation procedure using 2'-O-methyl-5'-O-DMT nucleosides with pyridinium trifluoroacetate

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To a sample of 2'-O-methyl-5'-O-DMT-nucleoside (2'-O-methyl-5'-O-DMT-6-N-benzoyladenosine, 2'-O-methyl-5'-O-DMT-4-N-benzoylcytidine, 2'-O-methyl-5'-O-DMT-2-N-isobutyrylguanosine, 2'-O-methyl-5'-O-DMT-thymidine and 2'-O-methyl-5'-O-DMT-uridine) (2a-2e, Figure 1, 1 mmol, 560 - 670 mg) in dry dichloromethane (3 mL) was added bisamidite reagent (0.38 mL, 362 mg, 1.2 mmol) at ambient temperature under argon. Pyridinium trifluoroacetate (232 mg, 1.2 mmol) was added to the reaction flask and the reaction mixture was stirred at ambient temperature for 2-3 hours. The reaction mixture was transferred directly to the top of a short silica gel column. The amidite product was eluted with 60-80% EtOAc/hexanes (1% triethylamine) with the exact concentration dependent to the respective amidite being purified. The appropriate fractions were collected and evaporated to give the respective amidite product (7a-e, Figure 1) as a white foam in 75-94% yield.

Example 3

20 General phosphitylation procedure using 2'-O-TBDMS-5'-O-DMT nucleosides with pyridinium trifluoroacetate

To a sample of 2'-O-TBDMS-5'-O-DMT-nucleoside (2'-O-TBDMS-5'-O-DMT-6-N-benzoyladenosine, 2'-O-TBDMS-5'-O-DMT-4-N-benzoylcytidine, 2'-O-TBDMS-5'-O-DMT-2-N-isobutyrylguanosine, 2'-O-TBDMS-5'-O-DMT-thymidine and 2'-O-TBDMS-5'-O-DMT-uridine) (3a-3e, Figure 1, 1 mmol, 661 - 770 mg) in dry dichloromethane (3 mL) was added bisamidite reagent (0.38 mL, 362 mg, 1.2 mmol) at ambient temperature under argon. Pyridinium trifluoroacetate (232 mg, 1.2 mmol) was added to the reaction flask and the reaction mixture was stirred at ambient temperature for 2-3 hours. The reaction solution was transferred directly to the top of a short silica gel column. The amidite product was eluted with 45-60% EtOAc/hexanes (1% triethylamine) with the exact concentration dependent to the respective amidite being

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purified. The appropriate fractions were collected and evaporated to give the respective amidite product (8a-e, Figure 1) as a white foam in 82-95% yield.

5 Example 4

General phosphitylation procedure using 2'-O-methoxyethyl-5'-O-DMT nucleosides with pyridinium trifluoroacetate

To a sample of 2'-O-(2-methoxyethyl)-5'-O-DMT-nucleoside (2'-O-(2-methoxyethyl)-5'-O-DMT-6-N-benzoyladenine, 2'-O-(2-methoxyethyl)-5'-O-DMT-4-N-benzoylcytosine, 2'-O-(2-methoxyethyl)-5'-O-DMT-2-N-isobutyrylguanosine, 2'-O-(2-methoxyethyl)-5'-O-DMT-thymidine, 2'-O-(2-methoxyethyl)-5'-O-DMT-uridine and 5-methyl-2'-O-(2-methoxyethyl)-5'-O-DMT-4-N-benzoylcytosine) (4a-f, Figure 1, 1 mmol, 619 - 714 mg) in dry dichloromethane (3 mL) was added bisamidite reagent (0.38 mL, 362 mg, 1.2 mmol) at ambient temperature under argon. Pyridinium trifluoroacetate (232 mg, 1.2 mmol) was added to the reaction flask and the reaction mixture was stirred at ambient temperature for 2-3 h. The reaction solution was transferred directly to the top of a short silica gel column. The amidite product was eluted with 60-80% EtOAc/hexanes (1% triethylamine) with the exact concentration dependent to the respective amidite being purified. The appropriate fractions were collected and evaporated to give the respective amidite product (9a-f, Figure 1) as a white foam in 92-95% yield.

Example 5

30 General procedure for phosphitylation of nucleoside 2'-deoxy-5'-O-DMT-4-N-benzoylcytosine with Poly(4-vinyl pyridine hydrochloride) as an activator

Poly(4-vinyl pyridine hydrochloride) (Aldrich, 583 mg, ~ 6.5 mmol Cl/g) resin was washed with dry acetonitrile

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(10 mL x 2). Dry dichloromethane (15 mL) and bisamidite reagent (1.20 mL, 1.14 g, 3.79 mmol) were added to the resin at ambient temperature under argon. Then a sample of 2'-deoxy-5'-O-DMT-4-N-benzoylcytidine (2.0 g, 3.16 mmol) was added and the reaction mixture was shaken by a mechanical shaker for 2 hours. The reaction was filtered and the filtrate was evaporated, and the residue was purified on a short silica gel column. The amidite product was eluted with 60% EtOAc/hexanes (1% triethylamine). The appropriate fractions were collected and evaporated to give amidite product (6b, figure 1) as a white foam (369 mg, 14%).

³¹P NMR (CDCl₃) δ 149.34, 149.94.

Example 6

General procedure for phosphitylation of the 5'-O-position of 6-N-benzoyl-2'-deoxy-3'-O-levulinyladenosine using pyridinium trifluoroacetate

A sample of 6-N-benzoyl-2'-deoxy-3'-O-levulinyladenosine (10, Figure 1, 1 mmol, 453 mg) in dry dichloromethane (3 mL) was added bisamidite reagent (0.38 mL, 362 mg, 1.2 mmol) at ambient temperature under argon. Pyridinium trifluoroacetate (232 mg, 1.2 mmol) was added and the reaction mixture was stirred at ambient temperature for 2 hours. The reaction solution was transferred directly to the top of a short silica gel column. The amidite product was eluted with EtOAc (1% triethylamine). The appropriate fractions were collected and evaporated to give the amidite product (11, Figure 1) as a white foam (601 mg, 92%).

³¹P NMR (CDCl₃) δ 149.58.

Example 7

Comparative study of activator efficiency using a) pyridinium acetate, b) pyridinium monochloroacetate, c) pyridinium dichloroacetate and d) pyridinium

trichloroacetate

Four separate reactions were run to determine the efficiency of selected pyridinium salts to act as an activator in phosphitylating 2'-deoxy-5'-O-DMT-4-N-benzoylcytidine. The activator species were produced *in situ* by addition of 1.2 eq. of the corresponding acetic acid, mono-, di- or trichloroacetic acid (0.56 mmol) to dry dichloromethane (1.5 mL) followed by addition of 1.3 eq. of pyridine (0.049 mL, 0.61 mmol). Bisamidite reagent (0.177 mL, 0.56 mmol) and 2'-deoxy-5'-O-DMT-4-N-benzoylcytidine (300 mg, 0.47 mmol) were added and the reaction mixtures were stirred under argon at ambient temperature. The progress of the reactions was monitored by TLC. There was no measurable reaction seen with the use of acetic acid and a slow reaction by use of either mono- or trichloroacetic acid (reaction not finished after 6.5 hours). At 6.5 hours the reaction was almost complete when dichloroacetic acid was used.

Example 8**20 Phosphitylation using pyridinium dichloroacetate, synthesis of 2'-deoxy-5'-O-DMT-4-N-benzoylcytidine diisopropylamino-cyanoethoxyphosphoramidite**

Pyridinium dichloroacetate was prepared *in situ* by addition of 1.3 equivalents of pyridine (0.49 mL, 6.07 mmol) to dry dichloromethane (4 mL) followed by addition of 1.2 eq. of dichloroacetic acid (0.46 mL, 5.60 mmol). To this mixture was added bisamidite reagent (1.78 mL, 5.60 mmol) followed by the dropwise addition of 2'-deoxy-5'-O-DMT-4-N-benzoylcytidine (2.97 g, 4.67 mmol) dissolved in dry dichloromethane (6 mL). The reaction mixture was stirred under argon at ambient temperature for 2 hours and transferred directly to the top of a short silica gel column. The amidite product was eluted with 70%

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EtOAc/hexanes (1% triethylamine). The appropriate fractions were collected and evaporated to give 3.47 g (89%) of the title compound as a white foam.

^{31}P NMR (CDCl_3) δ 149.29, 149.88.

5 Example 9

Stability of 5'-O-DMT protecting group to reaction conditions, synthesis of 2'-deoxy-5'-O-DMT-4-N-benzoylcytidine diisopropylaminocyanoethoxyphosphoramidite

4-N-Benzoyl-2'-deoxy-5'-O-DMT-cytidine (1.77 g, 2.79 mmol) was dissolved in dry dichloromethane (4 mL) under argon at ambient temperature followed by addition of bisamidite reagent (1.06 mL, 3.35 mmol) and pyridinium trifluoroacetate (0.65 g, 3.35 mmol). The mixture was stirred under reflux for 5 hours with no measurable loss of DMT protecting group. Product formation was identified by tlc compared to a known solution of product.

Example 10

Preparation of amidites without base protection, synthesis of 2'-deoxy-5'-O-DMT-adenosine diisopropylaminocyanoethoxy-phosphoramidite

Pyridinium trifluoroacetate (353 mg, 1.83 mmol) was added to a mixture of 2'-deoxy-5'-O-DMT-adenosine (1g, 841 mg, 1.52 mmol) and bisamidite reagent (0.53 mL, 505 mg, 1.67 mmol) in dichloromethane (5 mL). Stirring was continued for one hour at ambient temperature under argon atmosphere. The reaction solution was loaded without further workup on a silica gel column and eluted using a gradient of from 60 to 100% EtOAc/hexanes (1% triethylamine). The appropriate fractions were collected and evaporated to give 6.0g of the title compound as a white foam (689 mg, 60%).

^{31}P NMR (CDCl_3) δ 149.26, 149.92.

Example 11

**Stability of glycosidic linkage to reaction conditions,
synthesis of 2'-deoxy-5'-O-DMT-6-N-benzoyladenosine
diisopropylaminocyanoethoxyphosphoramidite**

5 2'-deoxy-5'-O-DMT-6-N-benzoyladenosine Procedure
(500 mg, 0.76 mmol) was dissolved in dry dichloromethane (1
mL) under argon at ambient temperature followed by addition
of bisamidite reagent (0.266 mL, 252mg, 0.837 mmol) and
pyridinium trifluoroacetate (176 mg, 0.913 mmol). The
10 mixture was stirred at ambient temperature for 2 hours, and
then stirred under reflux for 1.5 hours with no measurable
loss of the DMT protecting group or the adenine base.
Product formation was identified by tlc compared to a known
solution of product. This example shows the stability of
15 the most labile glycosidic linkage of a nucleoside under the
reaction conditions using this activator.

Example 12

**Mechanistic study of phosphitylation using pyridinium
trifluoroacetate**

20 The mechanism of phosphitylation was investigated
using the activator pyridinium trifluoroacetate and the
nucleoside 5'-O-DMT-thymidine using a Varian 400 MHz NMR.
The first set of experiments were performed by studying the
chemical shift of phosphorus nuclei under various conditions
25 (Table 1). In a second set of experiments the chemical
shift of nitrogen nuclei of various species were studied
(Table 2).

 The presence or absence of a specific phosphorus
species was determined by recording ³¹P NMR of 5'-O-DMT-
30 thymidine, bisamidite reagent and pyridinium
trifluoroacetate in CD₃CN. The order of addition was altered
in each individual experiment to determine which species is
formed in the reaction mixture.

 In experiment no. 1 (Table 1) the chemical shift

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of the ^{31}P signal in bisamidite reagent (5) is measured to be at 125.8 ppm in CD_3CN . The activator pyridinium trifluoroacetate (B) is then added to the solution of the solution of 5 and the ^{31}P NMR was recorded. A new signal appeared at 158.8 ppm upon addition of B to 5, in addition to the original signal of 125.8 ppm. The peak at 158 is believed to be a protonated species of 5 which appears to be stable and formed quickly. Next, addition of 5'-O-DMT-thymidine (1d) to the mixture shifts the signals to 151.2 and 151.0 ppm, due to the formation of diastereoisomers.

In experiment no. 2 (Table 1) 5'-O-DMT-thymidine (1d) bisamidite reagent (B) were taken together in CD_3CN and the ^{31}P NMR was recorded. It is note worthy that B alone can not react because the reagent is not activated or protonated. Thus, the chemical shift remains unchanged at 125.8 ppm. Addition of activator B to the mixture immediately forms the desired amidite 6d with ^{31}P shifts of 151.2 and 151.0 ppm.

In another experiment, bisamidite reagent (5) was treated with an acid such as trifluoroacetic acid instead of activator B and the ^{31}P NMR was recorded. First, the color of the reaction mixture changed from clear to dark and second there was no signal at 158 ppm for the protonated species.

Table 1

<u>compound</u>	<u>^{31}P NMR, chemical shift ppm (multiplicity)</u>
<u>Exp. No 1</u>	
5	125.8 (s)
5+B	158.8 (s)
	125.8 (s)
5+B+1d	151.2, 151.0 (s+s)

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Exp. No 2

1d+5	125.8(s)
1d+5+B	151.2, 151.0 (s+s)
5	125.8(s)

Wherein 1d = 2'-O-deoxy-5'-O-DMT-thymidine
5 = 2-cyanoethyl-N,N,N',N'-tetraiso
propylphosphorodiamidite

10 B = pyridinium trifluoroacetate

The experimental data show that the protonation of the phosphitylating reagent is the first step during the reaction sequence and that the protonated form is stable.

15 Support for this conclusion comes from the lack of signals seen for a second activated species that could form from nucleophilic attack by the counterion on the active species or alternatively reaction with free pyridine released during the protonation step. It is further seen that the use of

20 trifluoroacetic acid alone results in degradation of the phosphitylating reagent. If an acid was all that was needed for activation of the phosphitylating reagent then the active phosphorous species should be formed anyway, with a possible following attack of the trifluoroacetate. These

25 results demonstrate that the counterion does not participate in the mechanism. In other words the pyridinium ion acts as a proton donor and does not interact with the active phosphorus species further.

In experiment no. 3, (Table 2) ¹⁵N-labeled pyridine

30 was used to further establish the role of the free pyridine formed during the activation of the phosphitylating reagent. It had previously been seen that a phosphorus species having pyridine acting to give nucleophilic assistance was not seen.

Table 2

<u>compound</u>	<u>¹⁵N NMR, chemical shift ppm</u>
-----------------	---

<u>Exp. No 3</u>	
------------------	--

12	-69.0
----	-------

5

12+13	-148.0
-------	--------

5+12+13	-68.5
---------	-------

1d+5+12+13	-71.74
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10

Wherein 1d = 2'-O-deoxy-5'-O-DMT-thymidine
5 = 2-cyanoethyl-N,N,N',N'-tetraiso-
propylphosphorodiamidite
12 = pyridine
13 = trifluoroacetic acid

15

The ¹⁵N-labeled pyridine alone gives a signal at -69.0 ppm. The *in situ* formation of the activator is performed by addition of the trifluoroacetic acid. A signal is seen for the activator (pyridinium trifluoroacetate) at -148.0 ppm. Next, addition of the bisamidite reagent (5) causes the signal of the pyridine to revert back to that of free pyridine as expected. Again, addition of 2'-O-deoxy-5'-O-DMT-thymidine (1d) which underwent phosphitylation did not change the free pyridine signal. In conclusion, pyridine reacts with the trifluoroacetic acid to form activator (B) that reacts with 5 to produce activated phosphitylating reagent. At this point the pyridine reverts back to free pyridine where it remains unchanged for the remainder of the phosphitylation reaction. The slight change in chemical shift (Table 2) after the addition of the other reagents is due to the extreme sensitivity of the nitrogen signal to the concentration.

Example 13**Determination of efficiency of selected activators**

In order to determine the efficiency of activators under a variety of conditions 55 experiments were performed using a wide range of different activators (see Figures 2 and 3). A variety of nucleosides were employed including 2'-deoxy and 2'-O-modified nucleosides (see Figure 1). Different solvents were also employed including a dichloromethane, acetonitrile, ethyl acetate and toluene to evaluate the rate of reaction.

Table 3

	<u>Cmpd.Act.</u>		<u>Ratio</u>	<u>Sol</u>	<u>Time</u>	<u>Yld.</u>	<u>³¹P NMR</u>	
	<u>I</u>	<u>II</u>	<u>(I:P[III]:II)</u>			<u>hrs.</u>	<u>(%)</u>	
15	1a	A	1 : 1.4 : 1.4	DCM	3	72	149.32,	149.43
	1a	H	1 : 1.2 : 1.2	DCM	3	54	149.32,	149.43
	1a	F	1 : 1.2 : 1.2	ACN	2	51	149.32,	149.43
	1a	F	1 : 0.7 : 1.2	ACN	4.5	52	149.32,	149.43
	1a	C	1 : 1.2 : 1	ACN	2	42	149.32,	149.43
20	1a	B	1 : 1.2 : 1.2	ACN	2	90	149.32,	149.43
	1a	B	1 : 1.2 : 1.2	DCM	2	68	149.32,	149.43
	1a	I	1 : 1.2 : 1.2	ACN	48	–	N/A	
	1a	I	1 : 1.2 : 1.2	DCM	48	–	N/A	
	1a	J	1 : 1.2 : 1.2	DCM	48	–	N/A	
25	1a	K	1 : 1.2 : 1.2	DCM	17.5	–	N/A	
	1a	L	1 : 1.2 : 1.2	ACN	17.5	–	N/A	
	1a	M	1 : 1.2 : 1.2	DCM	0.25	71	149.32,	149.43
	1a	N	1 : 1.2 : 1.2	DCM	3	35	149.32,	149.43
	1a	N	1 : 1.2 : 1.2	DCM	20	32	149.32,	149.43
30	1b	A	1: 1.2 : 1.2	DCM	4	87	149.29,	149.88
	1b	B	1: 1.2 : 1.2	ACN	5	74	149.29,	149.88
	1b	A	1: 1.2 : 1.2	ACN	5	60	149.29,	149.88
	1b	G	1: 1.2 : 1.2	ACN	24	44	149.29,	149.88
	1b	B	1: 1.2 : 1.2	EtOAc	6	73	149.29,	149.88

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	1b	B*	1: 1.2 : 1.2	EtOAc	7	50	149.29, 149.88
	1b	B	1: 1.2 : 1.2	DCM	1	93	149.29, 149.88
	1c	A	1: 1.2 : 1.2	DCM	3	89	148.39, 149.15
	1c	A	1: 1.2 : 1.2	ACN	20	-	N/A
5	1c	A	1: 1.2 : 1.2	tol	20	-	N/A
	1c	B	1: 1.2 : 1.2	ACN	20	80	148.39, 149.15
	1c	B	1: 1.2 : 1.2	EtOAc	3	66	148.39, 149.15
	1c	B	1: 1.2 : 1.2	DCM	3	74	148.39, 149.15
	1d	D	1: 2.0 : 1	DCM	2	70	149.14, 149.57
10	1d	E	1: 1.4 : 0.3	DCM	2	86	149.14, 149.57
	1d	D	1: 1.1 : 1	DCM	3	94	149.14, 149.57
	1d	O	1: 1.2 : 0.6	DCM	3	41	149.14, 149.57
	1d	A	1: 1.2 : 1.2	DCM	3	86	149.14, 149.57
	1d	B	1: 1.2 : 1.2	DCM	3	88	149.14, 149.57
15	1d	C	1: 1.2 : 1	DCM	3	78	149.14, 149.57
	1d	C	1: 1.2 : 1.2	DCM	3	87	149.14, 149.57
	1d	P	1: 1.2 : 1.2	DCM	3	si.	N/A
	2a	B	1: 1.2 : 1.1	DCM	0.75	75	150.94, 151.67
	3a	B	1: 1.2 : 1.1	DCM	0.75	95	150.60, 151.05
20	4a	B	1: 1.2 : 1.1	DCM	0.8	96	149.66, 151.59
	2b	B	1: 1.2 : 1.2	DCM	2	94	150.77, 151.35
	3b	B	1: 1.2 : 1.2	DCM	2	90	149.85, 150.72
	4f	B	1: 1.2 : 1.2	DCM	2	92	150.76, 150.82
	2c	B	1: 1.2 : 1.2	DCM	2	86	150.71, 150.95
25	3c	B	1: 1.2 : 1.2	DCM	2	82	149.43, 150.37
	4c	B	1: 1.2 : 1.2	DCM	2	94	150.23, 150.82
	2e	B	1: 1.2 : 1.2	DCM	2	88	150.86, 151.39
	3e	B	1: 1.2 : 1.2	DCM	2	84	150.22, 150.61
	4d	B	1: 1.2 : 1.2	DCM	3	95	150.69, 150.83
30	1f	B	1: 1.2 : 1.2	DCM	2	91	149.14, 149.67
	10	Q	1: 1.2 : 1.2	DCM	2	14	149.34, 149.94
	1b	R*	1: 1.2 : 1.2	DCM	2	-	N/A
	1b	S*	1: 1.2 : 1.2	DCM	2	sl.	N/A
	1b	T*	1: 1.2 : 1.2	DCM	2	89	149.29, 149.88
35	1b	U*	1: 1.2 : 1.2	DCM	2	sl.	N/A

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1g B 1: 1.1 : 1.2 DCM 1 60 149.26, 149.92

Note: I = compound (nucleoside)
 II = activator (Act.)
 P[III] = phosphitylating agent
5 (bisamidite reagent)

- = no reaction

* = *in situ*

Si. = silylation

Sl. = slow reaction

10 Cmpd. = compound, see Figure 1

Act. = activator

Sol. = solvent

DCM = dichloromethane

ACN = acetonitrile

15 EtOAc = ethyl acetate

tol = toluene

Activators A = pyridine hydrochloride
 B = Pyridinium trifluoroacetate
20 C = Pyridinium triflate
 D = tetrazole
 E = diisopropylammonium tetrazolide
 F = 4,5-dicyanoimidazole
 G = imidazole hydrochloride
25 H = imidazolium triflate
 I = aniline hydrochloride
 J = *p*-anisidinium trifluoroacetate
 K = *p*-toluidine hydrochloride
 L = *o*-toluidine hydrochloride
30 M = 2-amino-4,6-dimethylpyrimidine
 trifluoroacetate
 N = 1,10-phenanthroline trifluoroacetate
 O = chlorotrimethylsilane (TMSCl)
 P = 1-(trimethylsilyl)imidazole

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- Q = poly(4-vinylpyridine hydrochloride)
R = pyridinium acetate
S = pyridinium chloroacetate
T = pyridinium dichloroacetate
5 U = pyridinium trichloroacetate

**Preparation of Intersugar Linkages Using Pyridinium
Salt/Substituted Imidazole Actoivators**

Example 14

Synthesis of T-T phosphorothioate dimer:

- 10 100 milligram (4 mmole) of 5'-O-Dimethoxytritylthymidine bonded to CPG (controlled pore glass) through an ester linkage was taken in a glass reactor, and a dichloromethane solution of 2% dichloroacetic acid (volume/volume) was added to deprotect the 5'-hydroxyl
15 group. The product was washed with dichloromethane and then with acetonitrile. Then, a 0.2 M solution of 5'-O-(4,4'-dimethoxytrityl)thymidine-3'-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite) in acetonitrile and a 0.22 M solution of pyridinium trifluoroacetate and 0.11M solution
20 of 1-methylimidazole in acetonitrile was added, and reacted at room temperature for 5 minutes. The product was washed with acetonitrile, and then a 0.05 M solution of Beaucage reagent in acetonitrile was added and reacted at room temperature for 5 minutes. This sulfurization step was
25 repeated one more time for 5 minutes. The support was washed with acetonitrile and then a solution of acetic anhydride/lutidine/THF (1:1:8), and N-methyl imidazole/THF was added to cap the unreacted 5'-hydroxyl group. The product was washed with acetonitrile.
30 The carrier containing the compound was treated with 30% aqueous ammonium hydroxide solution for 90 minutes. The aqueous solution was filtered, concentrated under reduced pressure to give phosphorothioate dimer of T-T.

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Example 15**Synthesis of C-T phosphorothioate dimer:**

100 milligram (4 mmole) of 5'-O-Dimethoxytritylthymidine bonded to CPG (controlled pore
5 glass) through an ester linkage was taken in a glass reactor, and a dichloromethane solution of 2% dichloroacetic acid (volume/volume) was added to deprotect the 5'-hydroxyl group. The product was washed with acetonitrile. Then, a
0.2 M solution of N⁴-Benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-
10 deoxycytidine-3'-O-(2-cyanoethyl)-N,N-diisopropylphosphoramidite) in acetonitrile and a 0.22 M solution of pyridinium trifluoroacetate and 0.11 M solution of 1-methylimidazole in acetonitrile was added, and reacted at room temperature for 5 minutes. The product was washed
15 with acetonitrile, and then a 0.05 M solution of Beaucage reagent in acetonitrile was added and reacted at room temperature for 5 minutes. This sulfurization step was repeated one more time for 5 minutes. The support was washed with acetonitrile and then a solution of acetic
20 anhydride/lutidine/THF (1:1:8), and N-methyl imidazole/THF was added to cap the unreacted 5'-hydroxyl group. The product was washed with acetonitrile.

The carrier containing the compound was treated with 30% aqueous ammonium hydroxide solution for 90 minutes
25 and then incubated at 55°C for 12 hours. The aqueous solution was filtered, concentrated under reduced pressure and then treated at room temperature with 1.0 M solution of tetra-n-butyl ammonium fluoride in THF to give a phosphorothioate dimer of dC-T.

30 Example 16**Synthesis of 5'-TTTTTTT-3' phosphorothioate heptamer:**

50 milligram (2 mmole) of 5'-O-dimethoxytritylthymidine bound to CPG (controlled pore glass) through an ester linkage was taken up in a glass

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reactor, and a toluene solution of 3% dichloroacetic acid (volume/volume) was added to deprotect the 5'-hydroxyl group. The product was washed with acetonitrile. Then, a 0.2 M solution of 5'-O-(4,4'-dimethoxytrityl)thymidine-3'-O-
5 (2-cyanoethyl N,N-diisopropylphosphoramidite) in acetonitrile and a 0.22 M solution of pyridinium trifluoroacetate and 0.11 M solution of 1-methylimidazole in acetonitrile was added, and allowed to react at room temperature for 5 minutes. The product was washed with
10 acetonitrile, and then a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) was added and allowed to react at room temperature for 3 minutes. This sulfurization step was repeated one more time for 3 minutes. The support was washed with acetonitrile, and then a
15 solution of acetic anhydride/lutidine/THF (1:1:8), and N-methyl imidazole/THF was added to cap any unreacted 5'-hydroxyl group. The product was washed with acetonitrile.

This complete cycle was repeated five more times to produce the completely protected thymidine heptamer. The
20 carrier containing the compound was treated with 30% aqueous ammonium hydroxide solution for 90 minutes at room temperature. The aqueous solution was filtered, and concentrated under reduced pressure to give a phosphorothioate heptamer, TTTTTTT.

25 Example 17

Synthesis of 5'-d(GACT)-3' phosphorothioate tetramer:

50 milligram (2 mmole) of 5'-O-dimethoxytritylthymidine bound to CPG (controlled pore glass) through an ester linkage was taken up in a glass
30 reactor, and a toluene solution of 3% dichloroacetic acid in toluene (volume/volume) was added to deprotect the 5'-hydroxyl group. The product was washed with acetonitrile. Then, a 0.2 M solution of 5'-O-(4,4'-dimethoxytrityl)thymidine-3'-O-(2-cyanoethyl N,N-

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diisopropylphosphoramidite) in acetonitrile and a 0.22 M solution of pyridinium trifluoroacetate and 0.11 M solution of 1-methylimidazole in acetonitrile was added, and allowed to react at room temperature for 5 minutes. The product was washed with acetonitrile, and then a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) was added and allowed to react at room temperature for 3 minutes. This sulfurization step was repeated one more time for 3 minutes. The support was washed with acetonitrile and then a solution of acetic anhydride/lutidine/THF (1:1:8), and N-methyl imidazole/THF was added to cap the unreacted 5'-hydroxyl group. The product was washed with acetonitrile.

A solution of 3% dichloroacetic acid in toluene (volume/volume) was added to deprotect the 5'-hydroxyl group. The product was washed with acetonitrile. Then, a 0.2 M solution of N⁴-benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxycytidine-3'-O-(2-cyanoethyl N,N-diisopropylphosphoramidite) in acetonitrile and a 0.22 M solution of pyridinium trifluoroacetate and 0.11 M solution of 1-methylimidazole in acetonitrile was added, and allowed to react at room temperature for 5 minutes. The product was washed with acetonitrile, and then a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) was added and allowed to react at room temperature for 3 minutes. This sulfurization step was repeated one more time for 3 minutes. The support was washed with acetonitrile and then a solution of acetic anhydride/lutidine/THF (1:1:8), and N-methyl imidazole/THF was added to cap any unreacted 5'-hydroxyl group. The product was washed with acetonitrile.

A solution of 3% dichloroacetic acid in toluene (volume/volume) was added to deprotect the 5'-hydroxyl group. The product was washed with acetonitrile. Then, a 0.2 M solution of N⁶-benzoyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyadenosine-3'-O-(2-cyanoethyl N,N-diisopropylphosphoramidite) in anhydrous acetonitrile and a

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0.22 M solution of pyridinium trifluoroacetate and 0.11 M solution of 1-methylimidazole in acetonitrile was added, and allowed to react at room temperature for 5 minutes. The product was washed with acetonitrile, and then a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) was added and allowed to react at room temperature for 3 minutes. This sulfurization step was repeated one more time for 3 minutes. The support was washed with acetonitrile and then a solution of acetic anhydride/lutidine/THF (1:1:8), and N-methyl imidazole/THF was added to cap the unreacted 5'-hydroxyl group. The product was washed with acetonitrile.

A solution of 3% dichloroacetic acid in toluene (volume/volume) was added to deprotect the 5'-hydroxyl group. The product was washed with acetonitrile. Then, a 0.2 M solution of N²-isobutyryl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxyguanosine-3'-O-(2-cyanoethyl N,N-diisopropylphosphoramidite) in acetonitrile and a 0.22 M solution of pyridinium trifluoroacetate and 0.11 M solution of 1-methylimidazole in acetonitrile was added, and allowed to react at room temperature for 5 minutes. The product was washed with acetonitrile, and then a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) was added and allowed to react at room temperature for 3 minutes. This sulfurization step was repeated one more time for 3 minutes. The support was washed with acetonitrile and then a solution of acetic anhydride/lutidine/THF (1:1:8), and N-methyl imidazole/THF was added to cap any unreacted 5'-hydroxyl group. The product was washed with acetonitrile.

The carrier containing the compound was treated with 30% aqueous ammonium hydroxide solution for 90 minutes at room temperature and then incubated at 55°C for 24 hour. The aqueous solution was filtered, concentrated under reduced pressure to give a phosphorothioate tetramer of 5'-dG-dA-dC-T-3'.

Example 18

Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA-CAT-GCA-TT)-3' phosphorothioate 20-mer

The synthesis of the above sequence was performed on a
5 Pharmacia OligoPilot II Synthesizer on a 180 mmole scale
using the cyanoethyl phosphoramidites and Pharmacia's primar
support. Detritylation was performed using 3%
dichloroacetic acid in toluene (volume/volume). Activation
of phosphoramidites was done with a 0.22 M solution of
10 pyridinium trifluoroacetate and 0.11 M solution of 1-
methylimidazole. Sulfurization was performed using a 0.2 M
solution of phenylacetyl disulfide in acetonitrile:3-
picoline (1:1 v/v) for 2 minutes. At the end of synthesis,
the support was washed with acetonitrile, cleaved,
15 deprotected and purified in the usual manner.

Example 19

Synthesis of fully-modified 5'-d(GCC-CAA-GCT-GGC-ATC-CGT-CA)-3' phosphorothioate 20-mer

The synthesis of the above sequence was performed on a
20 Pharmacia OligoPilot II Synthesizer on a 180 μ mole scale
using the cyanoethyl phosphoramidites and Pharmacia's primar
support. Detritylation was performed using 3% dichloroacetic
acid in toluene (volume/volume). Activation of
phosphoramidites was done with a 0.22 M solution of
25 pyridinium trifluoroacetate and 0.11 M solution of 1-
methylimidazole. Sulfurization was performed using a 0.2 M
solution of phenylacetyl disulfide in acetonitrile:3-
picoline (1:1 v/v) for 2 minutes. At the end of synthesis,
the support was washed with acetonitrile, cleaved,
30 deprotected and purified in the usual manner.

Example 20

Synthesis of fully-modified 5'-d(GCG-TTT-GCT-CTT-CTT-CTT-GCG)-3' phosphorothioate 21-mer

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The synthesis of the above sequence was performed on a Pharmacia OligoPilot II Synthesizer on a 180 μ mole scale using the cyanoethyl phosphoramidites and Pharmacia's primar support. Detritylation was performed using 3% dichloroacetic acid in toluene (volume/volume). Activation of phosphoramidites was done with a 0.22 M solution of pyridinium trifluoroacetate and 0.11 M solution of 1-methylimidazole. Sulfurization was performed using a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) for 2 minutes. At the end of synthesis, the support was washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 21

Synthesis of fully-modified 5'-d(GTT-CTC-GCT-GGT-GAG-TTT-CA)-3' phosphorothioate 20-mer

The synthesis of the above sequence was performed on a Pharmacia OligoPilot II Synthesizer on a 180 μ mole scale using the cyanoethyl phosphoramidites and Pharmacia's primar support. Detritylation was performed using 3% dichloroacetic acid in toluene (volume/volume). Activation of phosphoramidites was done with a 0.22 M solution of pyridinium trifluoroacetate and 0.11 M solution of 1-methylimidazole. Sulfurization was performed using a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) for 2 minutes. At the end of synthesis, the support was washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 22

Synthesis of fully-modified 5'-d(TCC-GTC-ATC-GCT-CCT-CAG-GG)-3' phosphorothioate 20-mer

The synthesis of the above sequence was performed on a Pharmacia OligoPilot II Synthesizer on a 180 μ mole scale

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using the cyanoethyl phosphoramidites and Pharmacia's primar support. Detritylation was performed using 3% dichloroacetic acid in toluene (volume/volume). Activation of phosphoramidites was done with a 0.22 M solution of
5 pyridinium trifluoroacetate and 0.11 M solution of 1-methylimidazole. Sulfurization was performed using a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) for 2 minutes. At the end of synthesis, the support was washed with acetonitrile, cleaved,
10 deprotected and purified in the usual manner.

Example 23

Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA)-2'-methoxyethyl-(CAT-GCA-TT)-3' phosphorothioate 20-mer

The synthesis of the above sequence was performed on a
15 Milligen 8800 Synthesizer on a 282 mmole scale using the cyanoethyl phosphoramidites and Pharmacia's primar support. Detritylation was performed using 3% dichloroacetic acid in toluene (volume/volume). Activation of phosphoramidites was done with a 0.22 M solution of pyridinium trifluoroacetate
20 and 0.11 M solution of 1-methylimidazole. Sulfurization was performed using a 0.4 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) for 6 minutes. At the end of synthesis, the support was washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

25 Example 24

Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA)-2'-methoxyethyl-(CAT-GCA-TT)-3' phosphorothioate 20-mer

The synthesis of the above sequence was performed on a Pharmacia OligoPilot II Synthesizer on a 250 mmole scale
30 using the cyanoethyl phosphoramidites and Pharmacia's primar support. Detritylation was performed using 3% dichloroacetic acid in toluene (volume/volume). Activation of phosphoramidites was done with a 0.22 M solution of

pyridinium trifluoroacetate and 0.11 M solution of 1-methylimidazole. Sulfurization was performed using a 0.4 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) for 6 minutes. At the end of synthesis, the support was washed with acetonitrile, cleaved, deprotected and purified in the usual manner

Example 25

Synthesis of fully-modified 5'-d(GC^{me}C^{me}-C^{me}AA-GC^{me}T-GGC^{me})-2'-methoxyethyl-(AU^{me}C^{me}-C^{me}GU^{me}-C^{me}A)-3' phosphorothioate 20-mer

10 The synthesis of the above sequence was performed on a OligoPilot II on a 200 mmole scale using the cyanoethyl phosphoramidites and Pharmacia's primar support. Detritylation was performed using 3% dichloroacetic acid in toluene (volume/volume). Activation of phosphoramidites was
15 done with a 0.22 M solution of pyridinium trifluoroacetate and 0.11 M solution of 1-methylimidazole. Sulfurization was performed using a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) for 3 minutes. At the end of synthesis, the support was washed with acetonitrile,
20 cleaved, deprotected and purified in the usual manner.

Example 26

Synthesis of fully-modified 5'-d(TGG-TGG_TGG_TGG_TGG_TGG-T)-3' phosphorothioate 20-mer

25 In order to compare the extent of formation of (n+1)-mers during the oligonucleotide synthesis between the two activators, the following experiment was conducted:

The synthesis of the above sequence was performed on a OligoPilot I on a 30 mmole scale using the cyanoethyl phosphoramidites and Pharmacia's primar support.
30 Detritylation was performed using 3% dichloroacetic acid in toluene (volume/volume). Activation of phosphoramidites was done with a 0.22 M solution of pyridinium trifluoroacetate and 0.11 M solution of 1-methylimidazole. Sulfurization was

performed using a 0.2 M solution of phenylacetyl disulfide in acetonitrile:3-picoline (1:1 v/v) for 3 minutes. At the end of synthesis, the support was washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

- 5 The above synthesis was repeated with 0.45 M solution of 1H-tetrazole. At the end of synthesis, the support was washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

10 The oligonucleotides were analyzed by capillary gel electrophoresis. A comparison of the two electropherograms shows that the two activators perform at the same efficiency.

Preparation of Internucleotide Linkages Between 2'-substituted Nucleosides Using Imidazolium Triflate Activator

15 **Example 27**

Synthesis of pyridinium tetrafluoborate, pyrinium hexafluoro phosphate, imidazolium salt and benzimidazolium salt

Pyridinium tetrafluoborate is prepared according to the procedure described by Brill et al., *J. Am. Chem. Soc.*, 1991
20 113, 3972.

Pyridinium tetrafluoborate is ion-exchanged with sodium hexafluorophosphate to give pyridinium hexafluorophosphate.

Imidazolium triflate is prepared according to the procedure of Kataoka et al., *Nucleic Acids Symposium Series*,
25 1998, 37, 21-22).

Benzimidazolium triflate is synthesized according to the reported procedure of Hayakawa et al., *J. Org. Chem.*, 1996, 61, 7996-7997.

Example 28

30 **Synthesis of benzimidazolium tetrafluoroborate**

To a solution of benzimidazole (10g, 84.6 mmol) in dichloromethane (30 mL) is added dropwise tetrafluoroboric

acid as its etherate (85%, HBF_4 by volume, Aldrich Chemicals Co.) with stirring at 0°C . The reaction mixture is diluted with diethylether (100 mL) to precipitate the title compound. The title compound is filtered, washed with ether and
5 recrystallized from ether.

Example 29

Synthesis of imidazolium tetrafluoroborate

To a solution of imidazole (20 mmol) in dichloromethane (30 mL) at 0°C HBF_4 (20 mmol, 3.8g of a diethyl etherate) in
10 dichloromethane is added dropwise. The reaction mixture is diluted with diethyl ether (100 mL) to precipitate the title compound. It is then filtered, washed with ether and recrystallized from ether.

15 Example 30

Synthesis of imidazolium hexafluorophosphate

Hexafluorophosphoric acid (65% in water) is purchased from Fluka and evaporated with pyridine three times to concentrate. A solution of imidazole or benzimidazole (20
20 mmol) in ether (100 mL) is treated with 20 mmol of evaporated hexafluorophosphoric acid under stirring and at 0°C . After mixing the solution is evaporated and the slurry is treated with anhydrous ether. The salt is isolated by filtration, followed by washing with ether and drying in vacuo.

25 Example 31

Synthesis of nucleobase-protected amidite monomer units derived from 2'-MOE nucleoside precursors using imidazolium salts

The nucleosidic monomers having 2'-O-(methoxyethyl)
30 modification are treated with 2-cyanoethyl-N,N,N',N'-tetraisopropyl phosphorodiamidite (1.2 equivalents) and imidazolium salt or benzimidazolium salt (0.5 equivalent) in dry methylenechloride at ambient temperature for about 30-60

minutes. Reaction progress and formation of the respective amidite is monitored by tlc. This general procedure is used to convert selected 2'-O-methoxyethoxy (2'-O-MOE) protected nucleosides into the respective phosphoramidites. One
5 equivalent of selected nucleosides 2'-O-(MOE)-5'-O-DMT-6-N-benzoyladenosine, 2'-O-(MOE)-5'-O-DMT-4-N-benzoylcytidine, 2'-O-(MOE)-5'-O-DMT-N-2-isobutyrylguanosine, 2'-O-(MOE)-5'-O-DMT-5-methyluridine, 2'-O-(MOE)-5'-O-DMT-uridine, 2'-O-(MOE)-5'-O-DMT-5-methyl-4-N-benzoylcytidine in anhydrous
10 dichloromethane is treated with 2-cyanoethyl-N,N,N',N'-tetraisopropyl phosphorodiamidite (1.2 equivalents) and either imidazolium salt or benzimidazolium salt (0.5 equivalent) at ambient temperature for 30-60 minutes under argon. The reaction mixture is directly loaded onto a silica
15 gel column and the product eluted with a gradient of ethylacetate/hexane. Desired product for each respective amidite is identified by tlc and collected and concentrated. Purity is determined by ¹H and ³¹P NMR studies.

Example 32

20 Synthesis of nucleobase-unprotected amidite monomers derived from 2'-MOE nucleoside precursors using imidazolium salts

The nucleoside monomers without the protecting group for exocyclic amines are synthesized in a similar manner to the previous example. 2'-O-(MOE)-5'-O-DMT adenosine, 2'-O-(MOE)-
25 5'-O-DMT-cytidine, 2'-O-(MOE)-5'-O-guanosine, 2'-O-(MOE)-5'-O-DMT-5-methyluridine, 2'-O-(MOE)-5'-O-DMT-uridine, 2'-O-(MOE)-5'-O-DMT-2-aminoadenosine, 2'-O-(MOE)-5'-O-DMT-5-methylcytidine, in each case 1 equivalent, is taken in anhydrous methylenechloride/DMF mixture and treated with 2-
30 cyanoethyl-N,N,N',N'-tetraisopropyl phosphorodiamidite (1.5 equivalents) and one of the imidazolium salts or benzimidazolium salts (0.5 equivalents) at ambient temperature for 30-60 mins under argon. The reaction mixture

is evaporated, toluene is added and reevaporated and the residue is dissolved in anhydrous methylenechloride and eluted with ethylacetate solvent. The phosphoramidite products are pooled and characterized by ^{31}P NMR.

5 Example 33

Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA-CAT-GCA-TT) - 3' phosphorothioate 20-mer

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using
10 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of pyridinium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed
15 using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 34

Synthesis of fully-modified 5'-d(GCC-CAA-GCT-GGC-ATC-CGT-CA) - 20 3' phosphorothioate 20-mer

The above sequence is prepared using an Expedite (Millipore) Synthesizer on a 1 micromole scale using 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene
25 chloride. Phosphoramidites are activated with a 0.22 M solution of pyridinium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified
30 in the usual manner.

Example 35

Synthesis of fully-modified 5'-d(GCG-TTT-GCT-CTT-CTT-CTT-

GCG)-3' phosphorothioate 21-mer

The above sequence is prepared on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Phosphoramidites are activated with a 0.22 M solution of pyridinium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. After synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 36**Synthesis of fully-modified 5'-d(GTT-CTC-GCT-GGT-GAG-TTT-CA) - 3' phosphorothioate 20-mer**

The above sequence is prepared on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Phosphoramidites are activated with a 0.22 M solution of pyridinium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 37**Synthesis of fully-modified 5'-d(TCC-GTC-ATC-GCT-CCT-CAG-GG) - 3' phosphorothioate 20-mer**

The above sequence is prepared on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Phosphoramidites are activated with a 0.22 M solution of pyridinium tetrafluoroborate and 0.11 M solution

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of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

5 Example 38

Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA)-2'-O-(MOE)-(CAT-GCA-TT)-3' phosphorothioate 20-mer

The above sequence is prepared on a Millipore Expedite Synthesizer on a 1 micromole scale using the 2-cyanoethyl
10 phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Phosphoramidites are activated with a 0.22 M solution of pyridinium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage
15 reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 39

Synthesis of fully-modified 5'-d(GCC CAA GCT GGC)-2'-O-(MOE)-(ATC CCG TCA)-3' phosphorothioate 20-mer

The above sequence is prepared on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene
25 chloride. Phosphoramidites are activated with a 0.22 M solution of pyridinium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified
30 in the usual manner

Example 40

Synthesis of fully-modified 5'-d(GC^{me}C^{me}-C^{me}AA-GC^{me}T-GGC^{me})-2'-O-

(MOE) - (A^{mo}C^{mo}-C^{mo}GU^{mo}-C^{mo}A) -3' phosphorothioate 20-mer

The above sequence is prepared on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation
5 is performed using 3% dichloroacetic acid in methylene chloride. Phosphoramidites are activated with a 0.22 M solution of pyridinium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Beaucage reagent is used for phosphorothioate synthesis. At the end of synthesis, the
10 support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 41**Synthesis of 2'-O-MOE gapmers**

Stock solutions of 2'-O-MOE amidites (0.1 M) are made in
15 anhydrous acetonitrile and loaded onto an Expedite Nucleic Acid synthesis system (Millipore) to prepare oligonucleotides. Commercially available deoxyamidites (A, T, C and G, PerSeptive Biosystem) are also made into stock solutions (0.1 M) with anhydrous acetonitrile. All syntheses
20 are carried out in the DMT ON mode. For the coupling of the 2'-O-MOE amidites coupling time is extended to 10 minutes and this step is carried out twice. All other steps in the protocol supplied by Millipore are used except the extended coupling time (240 seconds). Activation of phosphoramidites
25 is done with a 0.22 M solution of pyridinium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Beaucage reagent is used for phosphorothioate synthesis. The overall coupling efficiencies are expected to be greater than 95%. The oligonucleotides are cleaved from the controlled
30 pore glass (CPG) supports and deprotected under standard conditions using concentrated aqueous NH₄OH (30%) at 55°C. 5'-O-DMT containing oligomers are then purified by reverse phase liquid chromatography (C-4, Waters, 7-8 x 300 mm, A=50

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mM triethylammonium acetate pH 1, B=100%CH₃CN, 5 to 60% B in 60 minutes). Detritylation with aqueous 80% acetic acid (1 mL, 30 min., room temperature), evaporation, followed by desalting by using Sephadese G-25 column will yield the oligonucleotides expectedly as foams. All oligomers are analyzed by CGE, HPLC and mass spectrometry.

2'-MOE GAPMERS

	Sequence 5'-3'	Backbone	Target
10	T*sT*sC*sT*sC*s GsCsCsCsGsCsTsCs C*sT*sC*sC*sT*sC*sC*	P=S	c-raf
	T*sT*sC*sT*sC*s GsCsTsGsGsTsGs AsGs T*sT*sT*sC*sA*	P=S	pkc-a
	T*oT*oC*oT*oC*s GsCsCsCsGsCsTsCs C*oT*oC*oC*oT*oC*oC*	P=O, P=S, P=O	c-raf
15	T*oT*oC*oT*oC*s GsCsTsGsGsTsGs AsGs T*oT*oT*oC*oA*	P=O, P=S,	pkc-a

* = 2'-O-MOE

C's are all 5-methyl substituted

s = phosphorothioate internucleotide linkages

20 o = phosphodiester internucleotide linkages

Example 42**Synthesis of uniformly modified 2'-modified oligonucleotide**

2-O-MOE amidites of A, ^{5m}C, G and T are dissolved in anhydrous acetonitrile to give 0.1 M solution. These solutions are loaded onto an Expedite Nucleic Acid Synthesis system (Millipore) to synthesize the oligonucleotides. Activation of phosphoramidites is done with a 0.22 M solution of pyridinium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. The coupling efficiencies are expected to be more than 90 %. All steps in the protocol supplied by Millipore are used except the activation step. Beaucage reagent (0.1 M in acetonitrile) is used as a sulfurizing agent. For diester synthesis, t-BuOOH is used as the oxidizing agent.

35 The oligomers are cleaved from the controlled pore glass (CPG) supports and deprotected under standard conditions

using concentrated aqueous NH_4OH (30%) at 55 °C. 5'-O-DMT containing oligomers are then purified by reverse phase high performance liquid chromatography (C-4, Waters, 7.8 x 300 mm, A = 50 mM triethylammonium acetate, pH -7, B = acetonitrile, 5 5-60% of B in 60 min., flow 1.5 mL/minute). Detritylation with aqueous 80% acetic acid and evaporation, followed by desalting in a Sephadex G-25 column will give the oligonucleotides. Oligonucleotides are analyzed by HPLC, CGE and Mass spectrometry.

10

Sequence	Target
5' T*sC*sT*s G*sA*sG*s T*sA*sG*s C*sA*sG*s A*sG*sG*s A*sG*sC*s T*sC* 3'	I C A M , P=S
5' T*C*T*G*A*G*T*A*G*C*A*G*A*G*G*A*G*C*T*C* 3'	I C A M , P=O

15 T* = 2'-O-MOE T, A* = 2'-O-MOE A, C* = 2'-O-MOE ^{5me}C, G* = 2'-O-MOE G

Examples 43-60 Oligonucleotide synthesis employing pyridinium hexafluorophosphate

Example 43

20 Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA-CAT-GCA-TT)-3' phosphorothioate 20-mer

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support.

25 Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of pyridinium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis,
30 the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 44

Synthesis of fully-modified 5'-d(GCC-CAA-GCT-GGC-ATC-CGT-CA)-

3' phosphorothioate 20-mer

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support.

- 5 Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of pyridinium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis,
10 the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 45**Synthesis of fully-modified 5'-d(GCG-TTT-GCT-CTT-CTT-CTT-GCG)-3' phosphorothioate 21-mer**

- 15 The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done
20 with a 0.22 M solution of pyridinium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

25 Example 46**Synthesis of fully-modified 5'-d(GTT-CTC-GCT-GGT-GAG-TTT-CA)-3' phosphorothioate 20-mer**

- The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using
30 the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of pyridinium hexafluorophosphate and

0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

5 Example 47

Synthesis of fully-modified 5'-d(TCC-GTC-ATC-GCT-CCT-CAG-GG) - 3' phosphorothioate 20-mer

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using
10 the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of pyridinium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Sulfurization is
15 performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 48

20 Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA) -2' -O- (MOE) - (CAT-GCA-TT) -3' phosphorothioate 20-mer

The synthesis of the above sequence is performed on a Millipore Expedite Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in
25 methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of pyridinium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected
30 and purified in the usual manner.

Example 49

Synthesis of fully-modified 5'-d(GCC CAA GCT GGC) -2' -O- (MOE) -

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(ATC CCG TCA)-3' phosphorothioate 20-mer

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support.

- 5 Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of pyridinium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis,
- 10 the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner

Example 50**Synthesis of fully-modified 5'-d(GC^{me}C^{me}-C^{me}AA-GC^{me}T-GGC^{me})-2'-O-(MOE)-(AU^{me}C^{me}-C^{me}GU^{me}-C^{me}A)-3'-phosphorothioate 20-mer**

- 15 The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done
- 20 with a 0.22 M solution of pyridinium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Beaucage reagent is used for phosphorothioate synthesis. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

25 Example 51**Synthesis of 2'-MOE gapmers**

- A 0.1 M solution of 2'-O-MOE amidites are made in anhydrous acetonitrile and loaded onto an Expedite Nucleic Acid synthesis system (Millipore) to synthesize
- 30 oligonucleotides. All other deoxyamidites (A, T, C and G, PerSeptive Biosystem) used in synthesis are also made as 0.1 M solution in anhydrous acetonitrile. All syntheses are

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carried out in DMT on mode. For the coupling of the 2'-O-MOE
 amidites coupling time is extended to 10 minutes and this
 step is carried out twice. All other steps in the protocol
 supplied by Millipore are used except the extended coupling
 5 time (240 seconds). Activation of phosphoramidites is done
 with a 0.22 M solution of pyridinium hexafluorophosphate and
 0.11 M solution of 1-methylimidazole. Beaucage reagent is
 used for phosphorothioate synthesis. The overall coupling
 efficiencies are expected to be more than 95%. The
 10 oligonucleotides are cleaved from the controlled pore glass
 (CPG) supports and deprotected under standard conditions
 using concentrated aqueous NH_4OH (30%) at 55°C. 5'-O-DMT
 containing oligomers are then purified by reverse phase
 liquid chromatography (C-4, Waters, 7-8 x 300 mm, A=50 mM
 15 triethylammonium acetate pH 1, B=100% CH_3CN , 5 to 60% B in 60
 minutes). Detritylation with aqueous 80% acetic acid (1 mL,
 30 min., room temperature), concentration, followed by
 desalting by using Sephadese G-25 column will give the
 oligonucleotides as a pure foam. All oligomers are then
 20 analyzed by CGE, HPLC and mass spectrometry.

MOE GAPMERS

	Sequence 5'-3'	Backbone	Target
	T*sT*sC*sT*sC*s GsCsCsGsCsTsCs	P=S	c-raf
25	C*sT*sC*sC*sT*sC*sC*		
	T*sT*sC*sT*sC*s GsCsTsGsGsTsGs AsGs T*sT*sT*sC*sA*	P=S	pkc-?
	T*oT*oC*oT*oC*s GsCsCsGsCsTsCs	P=O, P=S, P=O	c-raf
30	C*oT*oC*oC*oT*oC*oC*		
	T*oT*oC*oT*oC*s GsCsTsGsGsTsGs AsGs T*oT*oT*oC*oA*	P=O, P=S,	pkc-?

* = 2'-O-MOE; All C = 5-methyl C;

Example 52**General procedure for uniformly modified 2'-modified
oligonucleotide synthesis**

2-O-MOE amidites of A, ^{5me}C, G and T are dissolved in
 5 anhydrous acetonitrile to give 0.1 M solution. These
 solutions are loaded onto an Expedite Nucleic Acid Synthesis
 system (Millipore) to synthesize the oligonucleotides.
 Activation of phosphoramidites is done with a 0.22 M solution
 of pyridinium hexafluorophosphate and 0.11 M solution of 1-
 10 methylimidazole. The coupling efficiencies are expected to be
 more than 95%. For the coupling of the first amidite
 coupling time is extended to 6 minutes and this step is
 carried out twice. All other steps in the protocol supplied
 by Millipore are used except the extended coupling time.
 15 Beaucage reagent (0.1 M in acetonitrile) is used as a
 sulfurizing agent. For diester synthesis, t-BuOOH is used as
 the oxidizing agent. The oligomers are cleaved from the
 controlled pore glass(CPG) supports and deprotected under
 standard conditions using concentrated aqueous NH₄OH (30%) at
 20 55 °C. 5'-O-DMT containing oligomers are then purified by
 reverse phase high performance liquid chromatography (C-4,
 Waters, 7.8 x 300 mm, A = 50 mM triethylammonium acetate, pH
 -7, B = acetonitrile, 5-60% of B in 60 min., flow 1.5
 mL/minute). Detritylation with aqueous 80% acetic acid and
 25 evaporation, followed by desalting in a Sephadex G-25 column
 will give the oligonucleotides. Oligonucleotides are analyzed
 by HPLC, CGE and Mass spectrometry.

Sequence	Target
5' T*sC*sT*s G*sA*sG*s T*sA*sG*s C*sA*sG*s A*sG*sG*s A*sG*sC*s T*sC* 3'	I C A M , P=S
5' T*C*T*G*A*G*T*A*G*C*A*G*A*G*G*A*G*C*T*C* 3'	I C A M , P=O

T* = 2'-O-MOE T, A* = 2'-O-MOE A, C* = 2'-O-MOE ^{5me}C, G* = 2'-
 O-MOE G

EXAMPLES 53-60

Oligonucleotide synthesis using benzimidazolium or imidazolium tetrafluoroborate activator

Example 53

5 **Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA-CAT-GCA-TT) - 3' phosphorothioate 20-mer**

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support.

- 10 Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the
15 end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 54

Synthesis of fully-modified 5'-d(GCC-CAA-GCT-GGC-ATC-CGT-CA) - 3'-phosphorothioate 20-mer

- 20 The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done
25 with a 0.22 M solution of imidazolium or benzimidazolium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

30 **Example 55**

Synthesis of fully-modified 5'-d(GCG-TTT-GCT-CTT-CTT-CTT-

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GCG)-3' phosphorothioate 21-mer

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support.

- 5 Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the
10 end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 56**Synthesis of fully-modified 5'-d(GTT-CTC-GCT-GGT-GAG-TTT-CA)-3' phosphorothioate 20-mer**

- 15 The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done
20 with a 0.22 M solution of imidazolium or benzimidazolium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

25 Example 57**Synthesis of fully-modified 5'-d(GCC CAA GCT GGC)-2'-O-(MOE)-(ATC CCG TCA)-3' phosphorothioate 20-mer**

- The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using
30 the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium

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tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

5 Example 56

Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA)-2'-O-(MOE)-(CAT-GCA-TT)-3' phosphorothioate 20-mer

The synthesis of the above sequence is performed on a Millipore Expedite Synthesizer on a 1 micromole scale using
10 the 2-cyanoethyl phosphoramidites and CPG support.

Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium tetrafluoroborate and 0.11 M solution of 1-methylimidazole.

15 Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 57

Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA)-2'-O-(MOE)-(CAT-GCA-TT)-3' phosphorothioate 20-mer

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using
20 the 2-cyanoethyl phosphoramidites and CPG support.

Detritylation is performed using 3% dichloroacetic acid in
25 methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium tetrafluoroborate and 0.11 M solution of 1-methylimidazole.

Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile,
30 cleaved, deprotected and purified in the usual manner

Example 58

Synthesis of fully-modified 5'-d(GC^{me}C^{me}-C^{me}AA-GC^{me}T-GGC^{me})-2'-O-

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(MOE) - (A^{mo}C^{mo}-C^{mo}GU^{mo}-C^{mo}A) - 3' phosphorothioate 20-mer

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support.

- 5 Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Beaucage reagent is used for phosphorothioate synthesis. At
10 the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 59**Synthesis of 2'-MOE gapmers**

- 15 A 0.1 M solution of 2'-O-MOE amidites are prepared in anhydrous acetonitrile and loaded onto an Expedite Nucleic Acid synthesis system (Millipore) to synthesize oligonucleotides. All other deoxyamidites (A, T, C and G, PerSeptive Biosystem) used in synthesis also made as 0.1 M
20 solution in anhydrous acetonitrile. All syntheses are carried out in DMT on mode. For the coupling of the 2'-O-MOE amidites coupling time is extended to 10 minutes and this step is carried out twice. All other steps in the protocol supplied by Millipore are used except the extended coupling
25 time (240 seconds). Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. Beaucage reagent is used for phosphorothioate synthesis. The overall coupling efficiencies are expected to be more than
30 95%. The oligonucleotides are cleaved from the controlled pore glass (CPG) supports and deprotected under standard conditions using concentrated aqueous NH₄OH (30%) at 55°C. 5'-O-DMT containing oligomers are then purified by reverse

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phase liquid chromatography (C-4, Waters, 7-8 x 300 mm, A=50 mM triethylammonium acetate pH 1, B=100%CH₃CN, 5 to 60% B in 60 minutes). Detritylation with aqueous 80% acetic acid (1 mL, 30 min., room temperature), concentration, followed by
 5 desalting by using Sephadese G-25 column will give the oligonucleotides as pure foams. All oligomers are then analyzed by CGE, HPLC and mass spectrometry.

MOE GAPMERS

	Sequence 5'-3'	Backbone	Target
10	T*sT*sC*sT*sC*s GsCsCsGsCsTsCs C*sT*sC*sC*sT*sC*sC*	P=S	c-raf
	T*sT*sC*sT*sC*s GsCsTsGsGsTsGs AsGs T*sT*sT*sC*sA*	P=S	pkc-a
15	T*oT*oC*oT*oC*s GsCsCsGsCsTsCs C*oT*oC*oC*oT*oC*oC*	P=O, P=S, P=O	c-raf
	T*oT*oC*oT*oC*s GsCsTsGsGsTsGs AsGs T*oT*oT*oC*oA*	P=O, P=S,	pkc-a

20 * = 2'-O-MOE; All C=5-methyl C;

Example 60

Synthesis of uniformly modified 2'-modified oligonucleotide

2-O-MOE amidites of A, ⁵meC, G and T are dissolved in anhydrous acetonitrile to give 0.1 M solution. These
 25 solutions are loaded onto an Expedite Nucleic Acid Synthesis system (Millipore) to synthesize the oligonucleotides. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium tetrafluoroborate and 0.11 M solution of 1-methylimidazole. The coupling efficiencies
 30 are expected to be more than 90%. For the coupling of the first amidite coupling time is extended to 10 minutes and this step is carried out twice. All other steps in the protocol supplied by Millipore are used except the extended coupling time. Beaucage reagent (0.1 M in acetonitrile) is

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used as a sulfurizing agent. For diester synthesis, t-BuOOH is used as the oxidizing agent.

The oligomers are cleaved from the controlled pore glass (CPG) supports and deprotected under standard conditions using concentrated aqueous NH₄OH (30%) at 55 °C. 5'-O-DMT containing oligomers are then purified by reverse phase high performance liquid chromatography (C-4, Waters, 7.8 x 300 mm, A = 50 mM triethylammonium acetate, pH -7, B = acetonitrile, 5-60% of B in 60 min., flow 1.5 mL/minute). Detritylation with aqueous 80% acetic acid and evaporation, followed by desalting in a Sephadex G-25 column will give the oligonucleotides. Oligonucleotides are analyzed by HPLC, CGE and Mass spectrometry.

15	Sequence	Target
	5' T*sC*sT*s G*sA*sG*s T*sA*sG*s C*sA*sG*s A*sG*sG*s A*sG*sC*s T*sC* 3'	I C A M , P=S
	5' T*C*T*G*A*G*T*A*G*C*A*G*A*G*G*A*G*C*T*C* 3'	I C A M , P=O

T* = 2'-O-MOE T, A* = 2'-O-MOE A, C* = 2'-O-MOE ^{5me}C, G* = 2'-

20 O-MOE G

EXAMPLES 61-70

Oligonucleotide Synthesis with imidazolium or benzimidazolium hexafluorophosphate as activator

25

Example 61

Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA-CAT-GCA-TT)-3' phosphorothioate 20-mer

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium

hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

5 Example 62

Synthesis of fully-modified 5'-d(GCC-CAA-GCT-GGC-ATC-CGT-CA)-3' phosphorothioate 20-mer

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using
10 the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium hexafluorophosphate and 0.11 M solution of 1-methylimidazole.
15 Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 63

Synthesis of fully-modified 5'-d(GCG-TTT-GCT-CTT-CTT-CTT-20 GCG)-3' phosphorothioate 21-mer

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in
25 methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile,
30 cleaved, deprotected and purified in the usual manner.

Example 64

Synthesis of fully-modified 5'-d(GTT-CTC-GCT-GGT-GAG-TTT-CA)-

3' phosphorothioate 20-mer

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support.

- 5 Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the
10 end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 65**Synthesis of fully-modified 5'-d(TCC-GTC-ATC-GCT-CCT-CAG-GG) - 3' phosphorothioate 20-mer**

- 15 The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done
20 with a 0.22 M solution of imidazolium or benzimidazolium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

25 Example 66**Synthesis of fully-modified 5'-d(TCC-CGC-CTG-TGA) -2' -O- (MOE) - (CAT-GCA-TT) -3' phosphorothioate 20-mer**

- The synthesis of the above sequence is performed on a Millipore Expedite Synthesizer on a 1 micromole scale using
30 the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium

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hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner.

5 Example 67

Synthesis of fully-modified 5'-d(GCC CAA GCT GGC)-2'-O-(MOE)-(ATC CCG TCA)-3' phosphorothioate 20-mer

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using
10 the 2-cyanoethyl phosphoramidites and CPG support.

Detritylation is performed using 3% dichloroacetic acid in methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium hexafluorophosphate and 0.11 M solution of 1-methylimidazole.
15 Sulfurization is performed using Beaucage reagent. At the end of synthesis, the support is washed with acetonitrile, cleaved, deprotected and purified in the usual manner

Example 68

Synthesis of fully-modified 5'-d(GC^{me}C^{me}-C^{me}AA-GC^{me}T-GGC^{me})-2'-O-(MOE)-(AU^{me}C^{me}-C^{me}GU^{me}-C^{me}A)-3' phosphorothioate 20-mer
20

The synthesis of the above sequence is performed on an Expedite (Millipore) Synthesizer on a 1 micromole scale using the 2-cyanoethyl phosphoramidites and CPG support. Detritylation is performed using 3% dichloroacetic acid in
25 methylene chloride. Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Beaucage reagent is used for phosphorothioate synthesis. At the end of synthesis, the support is washed with
30 acetonitrile, cleaved, deprotected and purified in the usual manner.

Example 69**Synthesis of 2'-MOE gapmers**

A 0.1 M solution of 2'-O-MOE amidites are prepared in anhydrous acetonitrile and loaded onto an Expedite Nucleic Acid synthesis system (Millipore) to synthesize oligonucleotides. All other deoxyamidites (A, T, C and G, PerSeptive Biosystem) used in synthesis are also made as 0.1 M solution in anhydrous acetonitrile. All syntheses are carried out in DMT on mode. For the coupling of the 2'-O-MOE amidites coupling time is extended to 10 minutes and this step is carried out twice. All other steps in the protocol supplied by Millipore are used except the extended coupling time (240 seconds). Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. Beaucage reagent is used for phosphorothioate synthesis. The overall coupling efficiencies are expected to be more than 95%. The oligonucleotides are cleaved from the controlled pore glass (CPG) supports and deprotected under standard conditions using concentrated aqueous NH_4OH (30%) at 55°C. 5'-O-DMT containing oligomers are then purified by reverse phase liquid chromatography (C-4, Waters, 7-8 x 300 mm, A=50 mM triethylammonium acetate pH 1, B=100% CH_3CN , 5 to 60% B in 60 minutes). Detritylation with aqueous 80% acetic acid (1 mL, 30 min., room temperature), concentration, followed by desalting by using Sephadese G-25 column will give the oligonucleotides as pure foams. All oligomers are then analyzed by CGE, HPLC and mass spectrometry.

MOE GAPMERS

Sequence 5'-3'	Backbone	Target
T*sT*sC*sT*sC*s GsCsCsGsCsTsCs C*sT*sC*sC*sT*sC*sC*	P=S	c-raf
T*sT*sC*sT*sC*s GsCsTsGsGsTsGs	P=S	pkc-a

	AsGs T*sT*sT*sC*sA*		
	T*oT*oC*oT*oC*s GsCsCsCsGsCsTsCs C*oT*oC*oC*oT*oC*oC*	P=O, P=S, P=O	c-raf
5	T*oT*oC*oT*oC*s GsCsTsGsGsTsGs AsGs T*oT*oT*oC*oA*	P=O, P=S,	pkc-a

*=2'-O-MOE; All C=5-methyl C;

Example 70

General procedure for uniformly modified 2'-modified

10 oligonucleotide synthesis

2-O-MOE amidites of A, ^{5me}C, G and T are dissolved in anhydrous acetonitrile to give 0.1 M solution. These solutions are loaded onto an Expedite Nucleic Acid Synthesis system (Millipore) to synthesize the oligonucleotides.

- 15 Activation of phosphoramidites is done with a 0.22 M solution of imidazolium or benzimidazolium hexafluorophosphate and 0.11 M solution of 1-methylimidazole. The coupling efficiencies are expected to be more than 90%. For the coupling of the first amidite coupling time is extended to 10
- 20 minutes and this step is carried out twice. All other steps in the protocol supplied by Millipore are used except the extended coupling time. Beaucage reagent (0.1 M in acetonitrile) is used as a sulfurizing agent. For diester synthesis, t-BuOOH is used as the oxidizing agent.
- 25 The oligomers are cleaved from the controlled pore glass(CPG) supports and deprotected under standard conditions using concentrated aqueous NH₄OH (30%) at 55 °C. 5'-O-DMT containing oligomers are then purified by reverse phase high performance liquid chromatography (C-4, Waters, 7.8 x 300 mm,
- 30 A = 50 mM triethylammonium acetate, pH -7, B = acetonitrile, 5-60% of B in 60 min., flow 1.5 mL/minute). Detritylation with aqueous 80% acetic acid and evaporation, followed by desalting in a Sephadex G-25 column will give the oligonucleotides. Oligonucleotides are analyzed by HPLC, CGE

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and Mass spectrometry.

Sequence	Target
5' T*sC*sT*s G*sA*sG*s T*sA*sG*s C*sA*sG*s A*sG*sG*s A*sG*sC*s T*sC* 3'	I C A M , P=S
5' T*C*T*G*A*G*T*A*G*C*A*G*A*G*G*A*G*C*T*C* 3'	I C A M , P=O

T* = 2'-O-MOE T, A* = 2'-O-MOE A, C* = 2'-O-MOE ^{5me}C, G* = 2'-O-MOE G

Example 71**10 Oligonucleotide synthesis without amino group protection**

Fully-modified 5'-d(TCC-CGC-CTG-TGA-CAT-GCA-TT)-3' phosphorothioate 20 mer; fully modified 5'-d(GCC-CAA-GCT-GGC-ATC-CGT-CA)-3' phosphorothioate 20 mer; fully-modified 5'-d(GCG-TTT-GCT-GCT-CTT-CTT-CTT-GCG)-3' phosphorothioate 21 mer; fully-modified 5'-d(GTT-CTC-GCT-GGT-GAG-TTT-CA)-3' phosphorothioate 20 mer; fully-modified 5'-d(TCC-GTC-ATC-GCT-CCT-CAG-GG)-3' phosphorothioate 20 mer; fully-modified 5'-d(TCC-CGC-CTG-TGA)2'-O-(MOE)-(CAT-GCA-TT)-3' phosphorothioate 20 mer; fully-modified 5'-d(GCC CAA GCT GGC)-2'-O-(MOE)-(ATC CCG TCA)-3' phosphorothioate 20-mer; fully-modified 5'-d(GC^{me}C^{me}-C^{me}AA-GC^{me}T-GGC^{me})-2'-O-(MOE)-(AU^{me}C^{me}-C^{me}GU^{me}-C^{me}A)-3' phosphorothioate 20 mer; and gapmers are synthesized.

2'-MOE GAPMERS

Sequence 5'-3'	Backbone	Target
25 T*sT*sC*sT*sC*s GsCsCsGsCsTsCs C*sT*sC*sC*sT*sC*sC*	P=S	c-raf
T*sT*sC*sT*sC*s GsCsTsGsGsTsGs AsGs T*sT*sT*sC*sA*	P=S	pkc-a
30 T*oT*oC*oT*oC*s GsCsCsGsCsTsCs C*oT*oC*oC*oT*oC*oC*	P=O, P=S, P=O	c-raf
T*oT*oC*oT*oC*s GsCsTsGsGsTsGs AsGs T*oT*oT*oC*oA*	P=O, P=S,	pkc-a

35 * = 2'-O-MOE; All C = 5-methyl C;

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Uniformly 2'-modified oligomers

	Sequence	Target
	5' T*sC*sT*s G*sA*sG*s T*sA*sG*s C*sA*sG*s A*sG*sG*s A*sG*sC*s T*sC* 3'	I C A M , P=S
5	5' T*C*T*G*A*G*T*A*G*C*A*G*A*G*G*A*G*C*T*C* 3'	I C A M , P=O

T* = 2'-O-MOE T, A* = 2'-O-MOE A, C* = 2'-O-MOE ^{5me}C, G* = 2'-O-MOE G

Nucleobase unprotected 2'-O-MOE amidites of A, ^{5me}C, G and T and nucleobase unprotected 2'-deoxy amidites of A, C, G and T are dissolved in anhydrous acetonitrile to give 0.1 M solutions. These solutions are loaded onto an Expedite Nucleic Acid Synthesis system (Millipore) to synthesize the oligonucleotides. Activation of phosphoramidites is done with a 0.22 M solution of one of the following activators: pyridinium tetrafluoroborate, pyridinium hexafluoroborate, imidazolium tetrafluoroborate, benzimidazolium tetrafluoroborate, imidazolium hexafluorophosphate or benzimidazolium hexafluorophosphate. After the coupling, any nucleobase N-phosphitylated side product is reverted back by treatment with excess of benzimidazolium triflate in methanol at 25°C for 2 minutes before proceeding to oxidation. The coupling efficiencies are expected to be more than 90%. For the coupling of the first amidite coupling time is extended to 10 minutes and this step is carried out twice. All other steps in the protocol supplied by Millipore are used except the extended coupling time. Beaucage reagent (0.1 M in acetonitrile) is used as a sulfurizing agent. For diester synthesis, t-BuOOH is used as the oxidizing agent.

30 **EXAMPLES 72-80**

Oligonucleotides with bioreversible protecting groups present in phosphate: Building blocks and oligonucleotide synthesis

Example 72**General Procedures**

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All reagents and solvents are purchased from Aldrich Chemical CO. Flash chromatography is performed on silica gel (Baker 40um). Thin layer chromatography is performed on Kieselgel 60 F-254 glass plates from E. Merck and compounds are visualized with UV light and sulfuric acid-methanol spray followed by charring. Solvent systems used for thin-layer chromatography and flash chromatography are: A; ethyl acetate-hexanes 1:1. B; ethyl acetate-hexanes-TEA 2:3:0.5. ¹H and ³¹P spectra are recorded using a Gemini 200 Varian spectrometer. All reactions are performed under an argon atmosphere and solutions rotary evaporated at 35-45°C in vacuo using a vacuum pump-vacuum controller combination.

Example 73

2'-O-MOE-5'-O-(4,4'-dimethoxytrityl)-5-methyluridine (S-pivaloyl-2-thioethyl) bis[N,N-diisopropylphosphoramidite]

To a stirred and precooled solution of 2'-O-MOE-5'-O-(4,4'-dimethoxytrityl)-5-methyluridine (10g, 16 mmol) and diisopropylethylamine (2.7g, 21 mmol) in dry dichloromethane (200 mL) in an ice bath is added dropwise a solution of N,N-(diisopropylamino)chlorophosphine (5.2g, 19 mmol) in dry dichloromethane. The resulting mixture is stirred at room temperature for 55 minutes. Imidazolium triflate (8.0 mmol) is added and a solution of S-(2-hydroxyethyl)thiopivaloate (Tosquellas, G. et al. *Nucleic Acid Res.* 26, 2069, 1998) (3.4g, 21 mmol) in dry dichloromethane is added dropwise over a period of 15 minutes. The reaction mixture is further stirred for 20 hours at room temperature. At the end of this time, the mixture is diluted with dry CH₂Cl₂ (100 mL) and washed with NaHCO₃ (80 mL) and brine 3 times (100 mL) each, dried over MgSO₄ and evaporated to a foam. Flash chromatography using 1:1 Hexanes:EtOAc containing 0.5% triethylamine will yield the title compound.

Example 74

2'-O-MOE-5'-O-(4,4'-dimethoxytrityl)-5-methyluridine (S-acetyl-2-thioethyl) bis[N,N-diisopropylphosphoramidite] 2'-O-MOE-5'-O-DMT-5-methyluridine

- 5 A solution of 2'-O-MOE-5'-O-DMT-5-methyluridine (10g, 16mmol) and diisopropylethylamine (2.7g, 21mmol) in dry dichloromethane (200 mL) is cooled in an ice bath and stirred for 15 min. Added dropwise a solution of N,N-(diisopropylamino)chlorophosphine (5.2g, 19 mmol) in dry
- 10 CH₂Cl₂. The resulting mixture is stirred at room temperature for 45 minutes. Added imidazolium triflate (8.0 mmol) and a solution of S-(2-hydroxyethyl)-thioacetate (Tosquellas et al. *Nucleic Acids Res.* 26, 2069, 1998) freshly prepared (2.6g, 21 mmol) in dry CH₂Cl₂ in a periods of 10 minutes. The reaction
- 15 mixture is further stirred for 18 hr at RT. At the end of this time, the mixture is diluted with dry CH₂Cl₂ (100 mL) and washed with NaHCO₃ (60 mL) and brine 3 times (80 mL) each and dried over MgSO₄ and evaporated to a solid light yellow foam. Purified by flash chromatography using 1:1
- 20 Hexanes:EtOAc containing 0.5% triethylamine will yield the desired product.

Example 75

2'-deoxy-5'-O-dimethoxytrityl-adenosine-(S-pivaloyl-2-thioethyl) bis[N,N-diisopropylphosphoramidite]

- 25 To a cooled solution of 2'-deoxy-5'-O-dimethyltrityl-adenosine (7.3mmol) and diisopropylamine (1.22g, 9.5mmol) in dry dichloromethane (100ml) stirred in an ice bath, is added a solution of N,N-(diisopropylamino)chlorophosphine (2.33g, 8.76mmol) dropwise in dry CH₂Cl₂. The resulting mixture is
- 30 stirred at RT for 45 min. A solution of S-(2-hydroxyethyl) thiopivaloate (1.42g, 8.76mmol) and imidazolium triflate (3.65mmol) in dry CH₂Cl₂ is added in a periods of 10 min. The reaction mixture is stirred for 22hr at RT. The mixture

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is diluted with CH₂Cl₂ (50 mL) and washed with NaHCO₃ (15 mL) and brine (25 mL) dried over MgSO₄, filtered and evaporated the solvent to a light yellow foam. Purification is done by flash chromatography using Hexanes:EtOAc 1:3 containing 0.5% triethylamine, will yield the desired product.

Example 76

2'-deoxy-5'-O-dimethyltrityl-cytidine-(S-pivaloyl-2-thioethyl) bis[N,N-diisopropylphosphoramidite]

The title compound is prepared as per the procedure described in example 75.

Example 77

2'-deoxy-5'-O-dimethyltrityl-cytidine-(S-benzoyl-2-thioethyl) bis[N,N-diisopropylphosphoramidite]

The title compound is prepared as per the procedure described in example 75.

Example 78

2'-deoxy-5'-O-dimethyltrityl-guanosine-(s-pivaloyl-2-thioethyl) bis[N,N-diisopropylphosphoramidite]

The title compound is prepared as per the procedure described in example 75.

Example 79

2'-deoxy-5'-O-dimethoxytrityl-adenosine-(S-acetyl-2-thioethyl) bis[N,N-diisopropylphosphoramidite]

The title compound is prepared as per the procedure described in example 75.

Example 80

2'-deoxy-5'-O-dimethoxytrityl-cytidine-(S-acetyl-2-thioethyl) bis[N,N-diisopropylphosphoramidite]

The title compound is prepared as per the procedure described in example 75.

EXAMPLES 81-86

- 5 Oligonucleotides synthesis with the aid of (*S*-Pivaloyl 2-Mercaptoethyl) 3'-O-[(5'-O-(4,4'-Dimethoxytrityl)Thymidyl] *N,N*-Diisopropylphosphoramidite

Example 81

- (*S*-Pivaloyl 2-mercaptoethyl) 3'-O-[(5'-O-(4,4'-dimethoxytrityl)thymidyl] *N,N*-diisopropylphosphoramidite

- Bis(*N,N*-diisopropylamino)phosphorochloridite (267mg, 1 mmol) in CH₂Cl₂ (2.5 mL) is added to a stirred solution of *S*-pivaloyl 2-mercaptoethanol (162 mg, 1 mmol) and ethyldiisopropylamine (142 mg, 1.1 mmol) in CH₂Cl₂ (1 mL for 15 5 min) at -30°C. The mixture is allowed to warm to room temperature and is stirred for 30 minutes to give *S*-Pivaloyl-2-mercaptoethyl-*N,N,N',N'*-tetraisopropyl phosphorodiamidite. The volume of solution is adjusted to 4.0 mL, an aliquot (320 mL) is taken and added to dry 5'-O-(4,4'-dimethoxytrityl)- 20 thymidine (21.7 mg, 40 mmol). Anhydrous imidazolium triflate (0.45 M in MeCN; 71 mL, 32 mmol) is added, and the mixture is stirred for 40 minutes at room temperature. The reaction is quenched with aqueous NaHCO₃ (5%; 2 mL), diluted with saturated NaCl (5 mL) and extracted with benzene (3x10 mL). 25 The extracts are dried over Na₂SO₄ and evaporated *in vacuo*. The residue is dissolved in 50% aqueous MeCN and purified by reversed phase HPLC on a DeltaPak 15 mm C18 300 column (7.8x300 mm). Isocratic elution with 50% aqueous MeCN for 10 minutes and with 75% aqueous MeCN for 25 minutes at a flow 30 rate 5 mL min⁻¹ is applied. Fractions containing pure are collected, diluted with water (50 mL) and extracted with benzene (5x10 mL). Extracts are dried over Na₂SO₄ and evaporated *in vacuo* to give *S*-pivaloyl 2-mercaptoethyl 3'-O-

[(5'-O-(4,4'-dimethoxytrityl)thymidyl] *N,N*-diisopropylphosphoramidite.

Example 82

Oligonucleotide synthesis

5 2-(pivaloylthio)ethyl-undecathymidylates are assembled on an ABI 380B DNA Synthesizer using 2-cyanoethyl 3-(4,4'-dimethoxytrityloxy)-3-(2-nitrophenyl)ethyl phosphate, phosphoramidite chemistry, benzimidazolium triflate or imidazolium triflate as the activator, and 3*H*-1,2-benzodithiol-3-one 1,1-dioxide Beaucage reagent (0.1 M in MeCN) as a sulfur-transfer reagent. 5'-O-(4,4'-dimethoxytrityl)thymidyl 2-(pivaloylthio)ethyl *N,N*-diisopropylaminophosphite is employed as a building block. After the synthesis, the oligonucleotide is cleaved from the support photolytically (Guzaev et al.
10 *Biol. Med. Chem. Lett.* 8, 1123, 1998).

Deprotection and isolation of oligonucleotides

The 5'-DMTr protected oligonucleotide is isolated by HPLC (DeltaPak 15 μ C18 300 Å, 3.9x300 mm; 0.1 M NH₄OAc as buffer A, 0.05 M NH₄OAc in 75% aqueous MeCN as buffer B; a
20 linear gradient from 15 to 80 % B in 30 minutes at a flow rate 5.0 mL min⁻¹). The collected fractions are evaporated, treated with 80% aqueous AcOH for 20 minutes, and evaporated to dryness. The residue is desalted on the same column eluting first with 0.1 M NaOAc (10 minutes), then with water
25 (10 minutes) and finally eluting as a sodium salt with 50% aqueous MeCN (20 minutes) at a flow rate 5.0 mL min⁻¹.

Example 83

Dodeca[(2-pivaloylthio)ethyl 2'-O-(MOE)-5-methyluridyl phosphate]

30 The title compound is prepared on an ABI 380B synthesizer by using 0.1 M (2-pivaloylthio)ethyl 5'-O-(4,4'-

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dimethoxytrityl)-2'-O-(MOE)-5-methyluridyl *N,N*-diisopropylaminophosphite in MeCN, photolabile solid support from the previous example, 0.45 M imidazolium triflate as an activator, 0.5 M *t*-BuOOH in MeCN as an oxidizer, and 6 minutes coupling time. Upon completeness of the chain assembly (DMTr-Off synthesis) the solid support is dried on an oil pump, placed in a Pyrex test tube and suspended in 80% aqueous MeCN (3 mL). The suspension is degassed, placed in photochemical reactor, and irradiated for 30 minutes at room temperature. The tube is centrifuged, and supernatant is collected. A fresh portion of 80% aqueous MeCN is added. This procedure is repeated for 5 times until less than 4 OD of oligonucleotide material is released after irradiation for 30 minutes. The collected supernatants are diluted with water to get a solution in 30% aqueous MeCN, applied on an HPLC column (DeltaPak 15 μ C18 300 A, 3.9 x 300 mm), and chromatographed in a linear gradient from 25 to 80% MeCN in water for 40 minutes. The main peak is collected and evaporated *in vacuo* to afford the title compound. An aliquot (5 OD) of the obtained material is treated with concentrated aqueous ammonia (2 mL) for 8 hours at room temperature, evaporated to dryness, and re-dissolved in water (200 μ L). Analysis by capillary electrophoresis (CE) will reveal comigration with authentic sample of dodeca[2'-O-(MOE)-5-methyluridyl phosphate].

Example 84

Dodeca[(2-pivaloylthio)ethyl 2'-O-(MOE)-5-methyluridyl thiophosphate]

The title compound is prepared as described above except that 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (0.05 M in MeCN) is used on an oxidation step as a sulfur transfer reagent. Chromatography on the same column in a linear gradient from 70 to 100% MeCN in water will afford the title compound. After treatment with concentrated aqueous ammonia as above,

analysis by capillary electrophoresis (CE) will reveal comigration with authentic sample of dodeca[2'-O-(MOE)-5-methyluridyl thiophosphate].

Example 85

5 3'-O-Diglycolyl-5'-(4,4'-dimethoxytrityl)thymidine derivatized CPG

The solid support is prepared according to references Pon, R.T. and Yu, S., *Nucleic Acid Res.* 1997, 25, 3629-3635, and Mullah, B. and Andrus, A., *Tetrahedron Lett.*, 1997, 38, 10 5751-5754. 5'-(4,4'-Dimethoxytrityl)thymidine (1090 mg, 2.0 mmol), diglycolic anhydride (689 mg, 6.0 mmol), pyridine (10 mL) is stirred for 7 hours at room temperature. The mixture is quenched with water (2 mL) for 10 minutes and evaporated to an oil. The residue is dissolved in ethyl acetate (50 15 mL), washed with triethylammonium acetate (2 M aqueous, 5 x 10 mL), then with water (5 x 10 mL), dried over Na₂SO₄ and evaporated. The residue is dissolved in pyridine (10 mL), long chain alkyl amine Controlled Pore Glass (CPG, 3.0g) is added and the mixture is degassed in vacuo. N,N'- 20 diisopropylcarbodiimide (800 mg, 6.3 mmol) is added, and the mixture is shaken overnight at room temperature. The solid support is filtered out, treated with a mixture of acetic anhydride, N-methylimidazole, 2,6-lutidine and THF (1:1:2:16 v/v) for 30 minutes, filtered, washed on filter with 25 acetonitrile (5 x 10 mL) and dried on an oil pump. Efficiency of the derivatization is determined by dimethoxytrityl assay to show the loading which is expected to be about 60 $\mu\text{mol g}^{-4}$.

Example 86

30 Oligonucleotide synthesis

Chimerical oligothymidylates are assembled on an ABI 380B DNA Synthesizer using 5'-O-(4,4'-

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dimethoxytrityl)thymidine 3'-O-(carboxymethyloxy)acetate derivatized CPG (diglycolyl-T CPG)¹ (Scheme 1), phosphoramidite chemistry, and either commercial oxidizer for 3H-1,2-benzodithiol-3-one 1,1-dioxide (0.05 M in MeCN) as the sulfur-transfer reagent. Either 5'-O-(4,4'-dimethoxytrityl)thymidyl 2-(pivaloylthio)ethyl N,N'-diisopropylaminophosphite or 3'-O-[5-methyl-2-O-(MOE)-5'-O-(4,4'-dimethoxytrityl)uridyl] 2-(pivaloylthio)ethyl N,N'-diisopropylaminophosphite are employed for chain assembly to create 2-(pivaloylthio)ethyl triester internucleosidic moieties. After extensive washing with MeCN and drying the oligonucleotide is released from the solid support by treatment with 0.01 M K₂CO₃ in MeOH (2x5 mL and 2x20 mL for 1 and 15 μ mol syntheses, respectively). Each portion is passed back and forth through the column for 45 minutes, neutralized by passing through short column with Dowex 50Wx8 (PyH⁺; ca. 1 mL). The combined eluates are evaporated to dryness, co-evaporated with MeCN (10 mL), and dissolved in water. The obtained mixture consists of along with products of methanolysis of 2-(pivaloylthio)ethyl groups (ca. 1 to 1.5% of methanolysis per each group). Target oligonucleotide is isolated by RP HPLC on Delta Pak 15 μ m C18 300Å column (3.9 x 300 mm and 7.8 x 300 mm for 1 and 15 μ mol syntheses, respectively), using 0.1 M NH₄OAc as buffer A, 80% aq MeCN as buffer B, and a linear gradient from 0 to 100% B in 50 minutes at a flow rate 1.5 and 5 mL min⁻¹, respectively. Collected fractions are evaporated, redissolved in water and desalted by injecting onto the same column, then washing with water (10 minutes) and eluting an oligonucleotide as an ammonium salt with 50% aq MeCN (20 minutes). Homogeneity of oligonucleotides is characterized by RP HPLC, mass spectrometry and ³¹P NMR.

Example 87**Synthesis of Bioreversible (SATE) Oligonucleotides without exocyclic amine protection using the activators**

Synthetic oligonucleotides as shown in Example 71, with
5 (S-pivaloyl 2-mercaptoethyl)bioreversible phosphate
protecting groups for the internucleotide phosphate linkages,
are synthesized with the aid of (S-pivaloyl 2-mercaptoethyl)
3'-O-[(5'-O-(4,4'-dimethoxytrityl)thymidyl] N,N-diisopropyl-
phosphoramidite, (S-pivaloyl 2-mercaptoethyl) 3'-O-[(5'-O-
10 (4,4'-dimethoxytrityl)adenyl] N,N-Diisopropylphosphoramidite,
(S-pivaloyl 2-mercaptoethyl) 3'-O-[(5'-O-(4,4'-
dimethoxytrityl)cytidyl] N,N-diisopropylphosphoramidite, and
(S-pivaloyl 2-mercaptoethyl) 3'-O-[(5'-O-(4,4'-
dimethoxytrityl)guanyl] N,N-diisopropylphosphoramidite
15 without exocyclic amine protection. Oligonucleotides are
assembled on an ABI 380B DNA Synthesizer using 5'-O-(4,4'-
dimethoxytrityl)nucleoside 3'-O-(carboxymethyloxy)acetate
derivatized CPG (diglycolyl-Nucleoside- CPG), phosphoramidite
chemistry, 3H-1,2-benzodithiol-3-one 1,1-dioxide (0.05 M in
20 MeCN) as the sulfur-transfer reagent, t-BuOOH as the
oxidizing agent for phosphodiester linkages. One of the
following activators as a 0.22 M solution along with a 0.11 M
solution of N-methyl-imidazole in acetonitrile is used as the
activator: pyridinium tetrafluoroborate, pyridinium
25 hexafluorophosphate, imidazoliumtetrafluoroborate, imidazolium
hexafluorophosphate, benzimidazolium tetrafluoroborate, or
benzimidazolium hexafluorophosphate, imidazolium triflate, or
benzimidazolium triflate. The small amount of nucleobase N-
phosphitylated side product is reverted back to the free
30 nucleoside derivative by treating the reaction solid support
with excess of benzimidazolium triflate in methanol at
ambient temperature for 2-3 minutes. After completeness of
oligonucleotide synthesis, the column is washed with dioxane
(10 mL) to give pivaloyl-containing oligonucleosides still on

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the solid support. After extensive washing with MeCN and drying, the oligonucleotide is released from the solid support by treatment with 0.01 M K_2CO_3 in MeOH (2x5 mL and 2x20 mL for 1 and 15 μ mol syntheses, respectively). Each portion is passed back and forth through the column for 45 minutes, neutralized by passing through short column with Dowex 50Wx8 (PyH^+ ; ca. 1 mL). The combined eluates are evaporated to dryness, co-evaporated with MeCN (10 mL), and dissolved in water. The obtained mixture includes the products of methanolysis of 2-(pivaloylthio)ethyl groups (ca. 1 to 1.5% of methanolysis per each group). Target oligonucleotide is isolated by RP HPLC on Delta Pak 15 μ m C18 300Å column (3.9 x 300 mm and 7.8 x 300 mm for 1 and 15 μ mol syntheses, respectively), using 0.1 M NH_4OAc as buffer A, 80% aq MeCN as buffer B, and a linear gradient from 0 to 100% B in 50 minutes at a flow rate 1.5 and 5 mL min^{-1} , respectively. Collected fractions are evaporated, redissolved in water and desalted by injection onto the same column, then washing with water (10 minutes) and finally elution as the ammonium salt with 50% aq MeCN (20 minutes). Homogeneity of chimerical oligonucleotides is characterized by RP HPLC and capillary electrophoresis, and their structure is confirmed by mass spectrometry and ^{31}P NMR.

It is intended that each of the patents, applications, printed publications, and other published documents mentioned or referred to in this specification be herein incorporated by reference in their entirety.

Those skilled in the art will appreciate that numerous changes and modifications may be made to the preferred embodiments of the invention and that such changes and modifications may be made without departing from the spirit of the invention. It is therefore intended that the appended claims cover all such equivalent variations as fall within the true spirit and scope of the invention.

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What is claimed is:

1. A method for preparing a phosphitylated compound comprising the steps of:
providing a compound having a hydroxyl group; and
5 reacting said compound with a phosphitylating reagent in the presence of a pyridinium salt in a solvent under conditions of time, temperature and pressure effective to yield said phosphitylated compound.
2. The method of claim 1 wherein said compound is a
10 nucleoside.
3. The method of claim 2 wherein said compound is a 5'-O- protected nucleoside having a 3' hydroxyl group.
4. The method of claim 1 wherein said compound is a nucleoside dimer having a 3' or 5' hydroxyl group.
- 15 5. The method of claim 1 wherein said compound is an oligonucleotide or oligonucleotide analog having a 3' or 5' hydroxyl group.
6. The method of claim 2 wherein said nucleoside has a 5' or a 2' hydroxyl group.
- 20 7. The method of claim 1 wherein said phosphitylating reagent is 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite, bis(N,N-diisopropylamino)-2-methyltrifluoroacetylaminooethoxyphosphine or bis(N,N-diisopropylamino)-2-diphenylmethylsilylethoxyphosphine.
- 25 8. The method of claim 1 wherein said pyridinium salt is pyridinium hydrochloride, pyridinium trifluoroacetate or pyridinium dichloroacetate.

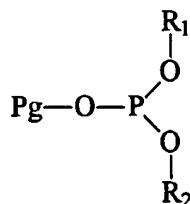
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9. The method of claim 1 wherein said solvent is dichloromethane, acetonitrile, ethyl acetate, tetrahydrofuran or a mixture thereof.

5 10. The method of claim 1, wherein said activator is bound to a solid support.

11. The method of claim 10 wherein said activator is a polyvinyl pyridinium salt.

10 12. A method for the preparation of a compound of formula:



wherein:

R_1 is a nucleoside or an oligonucleotide;

15 R_2 is a nucleoside linked to a solid support, or an oligonucleotide linked to a solid support;

Pg is a phosphorus protecting group;

comprising:

providing a phosphoramidite of formula:



wherein

R_6 is $-\text{N}(\text{R}_7)_2$ wherein R_7 is alkyl having from one to about six carbons; or R_7 is a heterocycloalkyl or heterocycloalkenyl ring containing from 4 to 7 atoms, and

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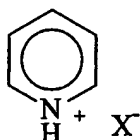
having up to 3 heteroatoms selected from nitrogen, sulfur, and oxygen; and

reacting said phosphoramidite with a hydroxyl group of a nucleoside linked to a solid support, or an oligonucleotide
5 linked to a solid support;

said reaction being performed in the presence of an activating reagent, said activating reagent comprising at least one pyridinium salt and at least one substituted imidazole.

10

13. The method of claim 12 wherein said pyridinium salt has the formula



where X⁻ is trifluoroacetate, ⁻O-mesyl, ⁻O-tosyl, ⁻Br,
15 ⁻O-trifluorosulfonyl, hexafluorophosphate or tetrafluoroborate.

14. The method of claim 13 wherein the substituted imidazole is 1-methylimidazole.

15. The method of claim 13 wherein X⁻ is
20 trifluoroacetate.

16. The method of claim 12 wherein the phosphoramidite is reacted with the 5'-hydroxyl of a solid-support bound nucleoside or oligonucleotide.

17. The method of claim 12 wherein the oligonucleotide
25 comprises phosphorothioate intersugar linkages.

18. A method for the preparation of an oligonucleotide

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comprising the steps of:

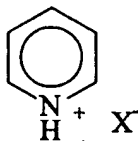
providing a 3'-nucleoside phosphoramidite or 3'-oligonucleotide phosphoramidite; and

reacting said 3'-nucleoside phosphoramidite or 3'-oligonucleotide phosphoramidite with the 5'-hydroxyl of a nucleoside, or oligonucleotide in the presence of an activating reagent;

said nucleoside or oligonucleotide being optionally bound to a solid support;

said activating reagent comprising at least one pyridinium salt and one substituted imidazole.

19. The method of claim 18 wherein the pyridinium salt has the formula



15 where X⁻ is trifluoroacetate, ⁻O-mesyl, ⁻O-tosyl, ⁻Br, ⁻O-trifluorosulfonyl, hexafluorophosphate or tetrafluoroborate.

20. The method of claim 19 wherein the substituted imidazole is 1-methylimidazole.

21. The method of claim 20 wherein X⁻ is trifluoroacetate.

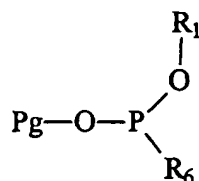
22. The method of claim 20 wherein the 3'-mononucleoside phosphoramidite or 3'-oligonucleotide phosphoramidite is reacted with the 5'-hydroxyl of a solid-support bound nucleoside, nucleotide or oligonucleotide.

23. The method of claim 20 wherein the oligonucleotide

- 126 -

comprises phosphorothioate intersugar linkages.

24. A synthetic method comprising:
providing a phosphoramidite of formula:



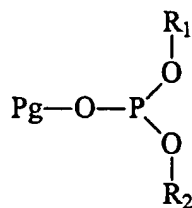
5 wherein:

R_1 is a nucleoside or an oligonucleotide;

R_6 is $-\text{N}(\text{R}_7)_2$ wherein R_7 is alkyl having from one to about six carbons; or R_7 is a heterocycloalkyl or heterocycloalkenyl ring containing from 4 to 7 atoms, and
10 having up to 3 heteroatoms selected from nitrogen, sulfur, and oxygen;

Pg is a phosphorus protecting group;

and reacting said phosphoramidite with a hydroxyl group of a nucleoside linked to a solid support, or an
15 oligonucleotide linked to a solid support, to form a compound of formula:



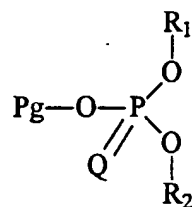
wherein:

R_2 is a nucleoside linked to a solid support, or an
20 oligonucleotide linked to a solid support;

said reaction being performed in the presence of an activating reagent, said activating reagent comprising at least one pyridinium salt and one substituted imidazole; and oxidizing or sulfurizing said compound to form a

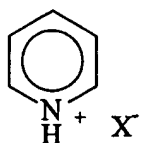
- 127 -

compound of formula:



wherein Q is O or S.

25. The method of claim 24 wherein the pyridinium salt
5 has the formula



where X⁻ is trifluoroacetate, ⁻O-mesyl, ⁻O-tosyl, ⁻Br, ⁻O-trifluorosulfonyl, hexafluorophosphate or tetrafluoroborate.

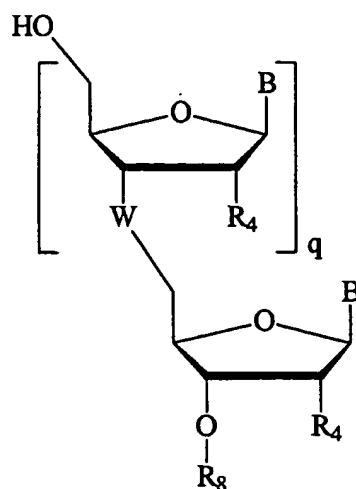
- 10 26. The method of claim 25 wherein the substituted imidazole is 1-methylimidazole.

27. The method of claim 26 wherein X⁻ is trifluoroacetate.

28. The method of claim 16 wherein Q is S.

- 15 29. A synthetic method comprising:
providing a compound of Formula X:

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X

wherein:

B is a nucleobase;

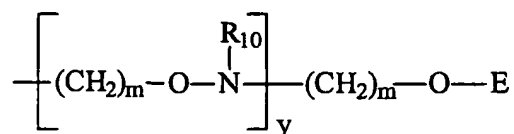
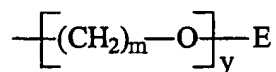
5 R_8 is H, a hydroxyl protecting group, or a linker connected to a solid support;

W is an optionally protected internucleoside linkage;

q is 0 to about 50;

R_4 is H, F, O-R, S-R or N-R(R_{10});

10 R is H, a protecting group, or has one of the formulas:



where

each m is independently from 1 to 10;

15 y is from 0 to 10;

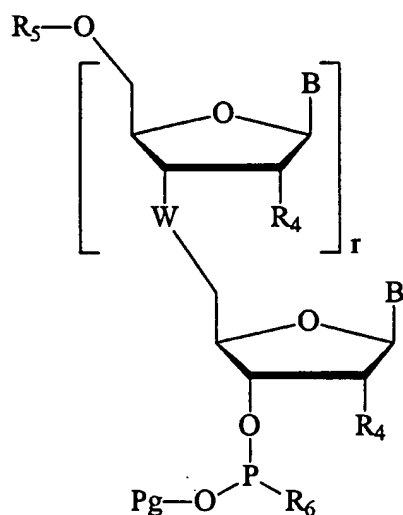
E is H, a hydroxyl protecting group, C_1 - C_{10} alkyl, $N(R_{10})(R_{11})$ or $N=C(R_{10})(R_{11})$; substituted or unsubstituted C_1 - C_{10} alkyl, C_2 - C_{10} alkenyl, C_2 - C_{10} alkynyl, wherein the substitutions are selected from one or several halogen,

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cyano, carboxy, hydroxy, nitro and mercapto residues; and
 each R_{10} or R_{11} is, independently, H, substituted or
 unsubstituted C_1 - C_{10} alkyl, C_2 - C_{10} alkenyl, C_2 - C_{10} alkynyl,
 wherein the substitutions are selected from one or several
 5 halogen, cyano, carboxy, hydroxy, nitro and mercapto
 residues; alkylthioalkyl, a nitrogen protecting group, or R_{10}
 and R_{11} , together, are a nitrogen protecting group or wherein
 R_{10} and R_{11} are joined in a ring structure that can include at
 least one heteroatom selected from N and O;

10 or R is $-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}_2-\text{N}(\text{R}_{10})(\text{R}_{11})$;

reacting the compound of Formula X in the presence of an
 activating reagent with a compound of Formula XI:



XI

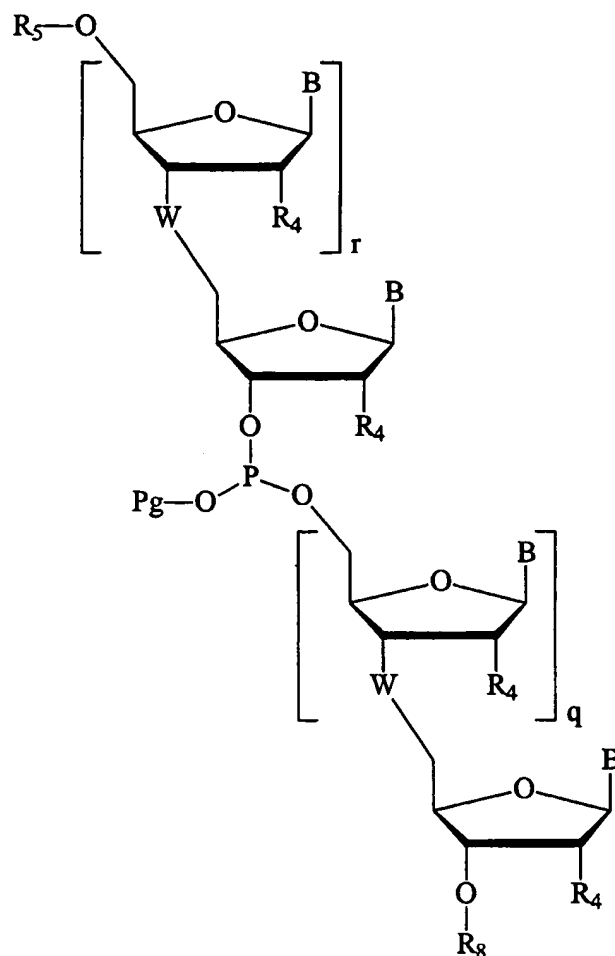
15

where r is 0 to about 50;

R_5 is a hydroxyl protecting group;

R_6 is $-\text{N}(\text{R}_7)_2$ wherein R_7 is alkyl having from one to
 about six carbons; or R_7 is a heterocycloalkyl or
 20 heterocycloalkenyl ring containing from 4 to 7 atoms, and
 having up to 3 heteroatoms selected from nitrogen, sulfur,
 and oxygen;

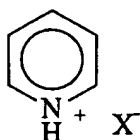
to form a compound of Formula XII:



XII

wherein said activating reagent comprises at least one pyridinium salt and one substituted imidazole.

- 5 30. The method of claim 29 wherein the pyridinium salt has the formula:



where X^- is trifluoroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br , or ^-O -trifluorosulfonyl, hexafluorophosphate or
 10 tetrafluoroborate.

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31. The method of claim 30 wherein the substituted imidazole is 1-methylimidazole.

32. The method of claim 31 wherein X⁻ is trifluoroacetate.

5 33. The method of claim 29 wherein R₈ is a linker connected to a solid support.

34. The method of claim 29 wherein R₄ is -O-R wherein R has the formula $[-(\text{CH}_2)_m-\text{O}]_y-\text{E}$; m is 2, y is 1; and E is CH₃, -N(R₁₀)(R₁₁), or -CH₂-CH₂-N(R₁₀)(R₁₁).

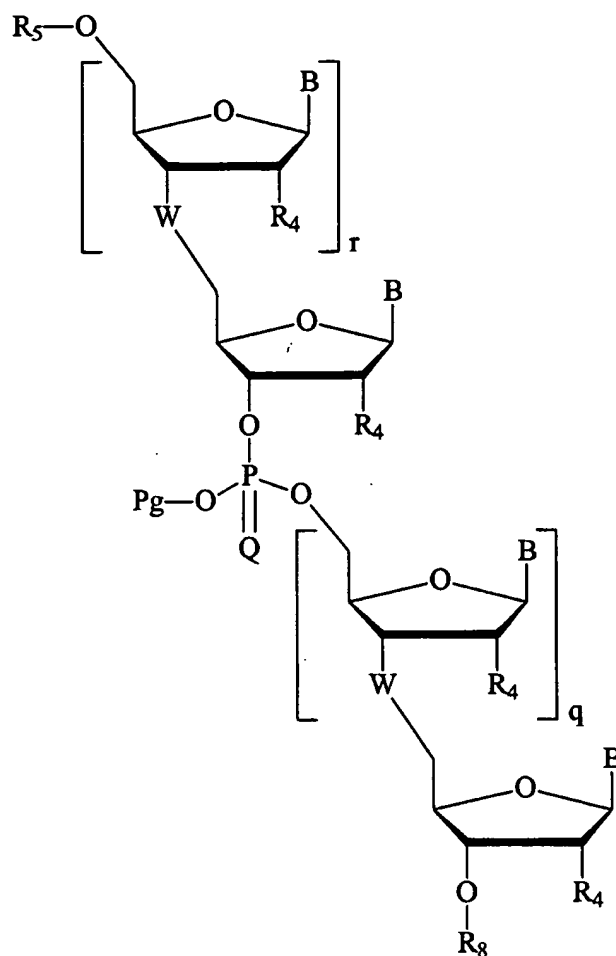
10 35. The method of claim 29 wherein r is 0.

36. The method of claim 29 wherein R₆ is diisopropylamino.

37. The method of claim 29 wherein Pg is -CH₂CH₂CN, -CH₂CH=CHCH₂CN, para-CH₂C₆H₄CH₂CN, -(CH₂)₂₋₅N(H)COCF₃,
15 -CH₂CH₂Si(C₆H₅)₂CH₃, or -CH₂CH₂N(CH₃)COCF₃.

38. The method of claim 29 wherein Pg is -CH₂CH₂CN.

39. The method of claim 29 further comprising oxidizing or sulfurizing the compound of Formula XII to form a compound of Formula XIII:



XIII

where Q is O or S.

40. The method of claim 39 further comprising a capping
5 step.

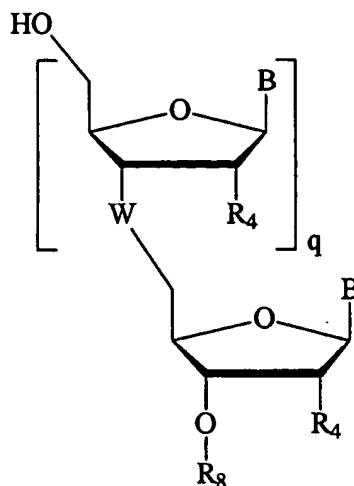
41. The method of claim 40 wherein the capping step is
performed prior to oxidation.

42. The method of claim 41 further comprising the step
of cleaving the oligomeric compound to produce a further
10 compound of formula X.

43. A synthetic method comprising:

- 133 -

providing a compound of Formula X:



X

5 wherein:

B is a nucleobase;

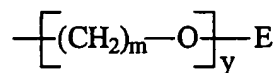
R₈ is H, a hydroxyl protecting group, or a linker connected to a solid support;

W is an optionally protected internucleoside linkage;

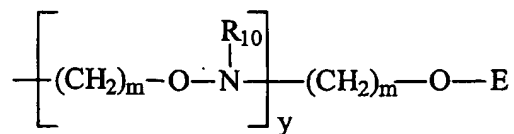
10 q is 0 to about 50;

R₄ is H, F, O-R, S-R or N-R(R₁₀);

R is H, a protecting group, or has one of the formulas:



15



where,

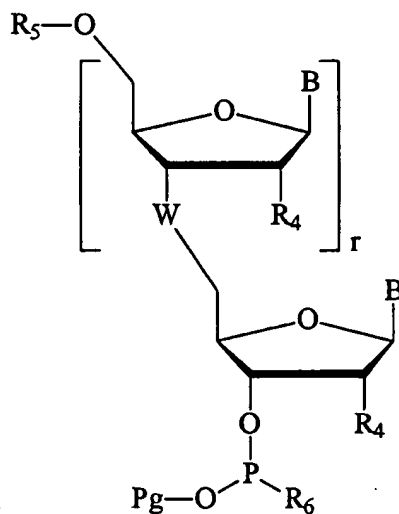
each m is independently from 1 to 10;

y is from 0 to 10;

E is H, a hydroxyl protecting group, C₁-C₁₀ alkyl,

20 N(R₁₀) (R₁₁) or N=C(R₁₀) (R₁₁); substituted or unsubstituted

C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, wherein the
 substitutions are selected from one or several halogen,
 cyano, carboxy, hydroxy, nitro and mercapto residues; and
 each R_{10} or R_{11} is, independently, H, substituted or
 5 unsubstituted C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl,
 wherein the substitutions are selected from one or several
 halogen, cyano, carboxy, hydroxy, nitro and mercapto
 residues; alkylthioalkyl, a nitrogen protecting group, or R
 and R_{10} , together, are a nitrogen protecting group or wherein
 10 R and R_2 are joined in a ring structure that can include at
 least one heteroatom selected from N and O;
 or R is $-CH_2-CH_2-O-CH_2-CH_2-N(R_{10})(R_{11})$;
 provided that R_{14} is not H or OH;
 reacting the compound of Formula X in the presence of an
 15 activator with a compound of Formula XI:

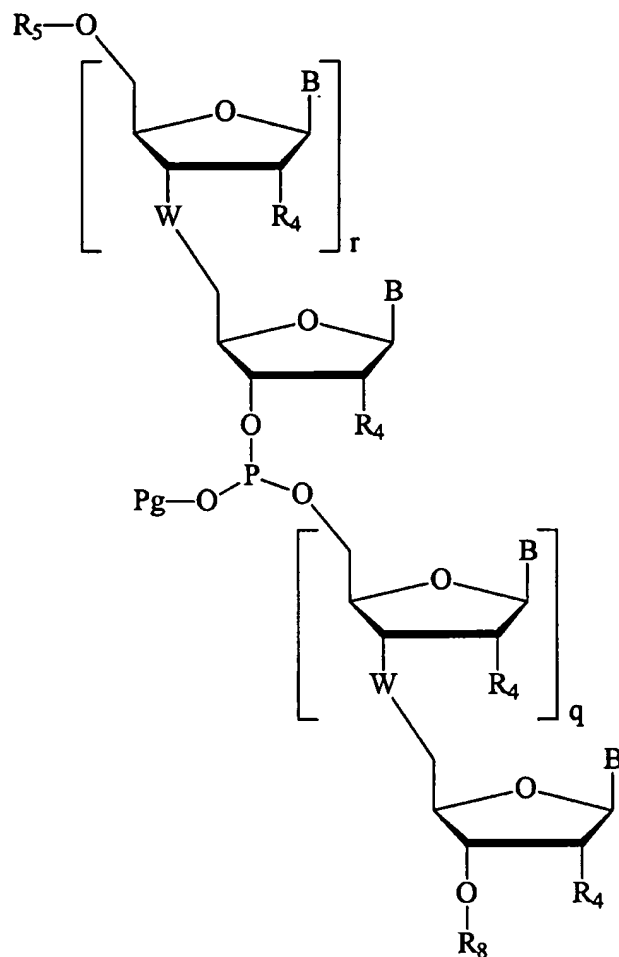


XI

where r is 0 to about 50;
 20 R_5 is a hydroxyl protecting group;
 R_6 is $-N(R_7)_2$ wherein R_7 is alkyl having from one to
 about six carbons; or R_7 is a heterocycloalkyl or
 heterocycloalkenyl ring containing from 4 to 7 atoms, and
 having up to 3 heteroatoms selected from nitrogen, sulfur,

and oxygen;

to form a compound of Formula XII:



XII

5 wherein the activator has the formula G^+U^- ,
 where

G^+ is selected from the group consisting of pyridinium, imidazolium, and benzimidazolium; and

10 U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate, triflate, hydrochloride, trifluoroacetate, dichloroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br , and ^-O -trifluorosulfonyl.

44. The method of claim 43 wherein R_8 is a linker

connected to a solid support.

45. The method of claim 44 wherein R_4 is $-O-R$ wherein R has the formula $-[-(CH_2)_m-O-]_y-E$; m is 2, y is 1; and E is CH_3 , $-N(R_{10})(R_{11})$, or $-CH_2-CH_2-N(R_{10})(R_{11})$.

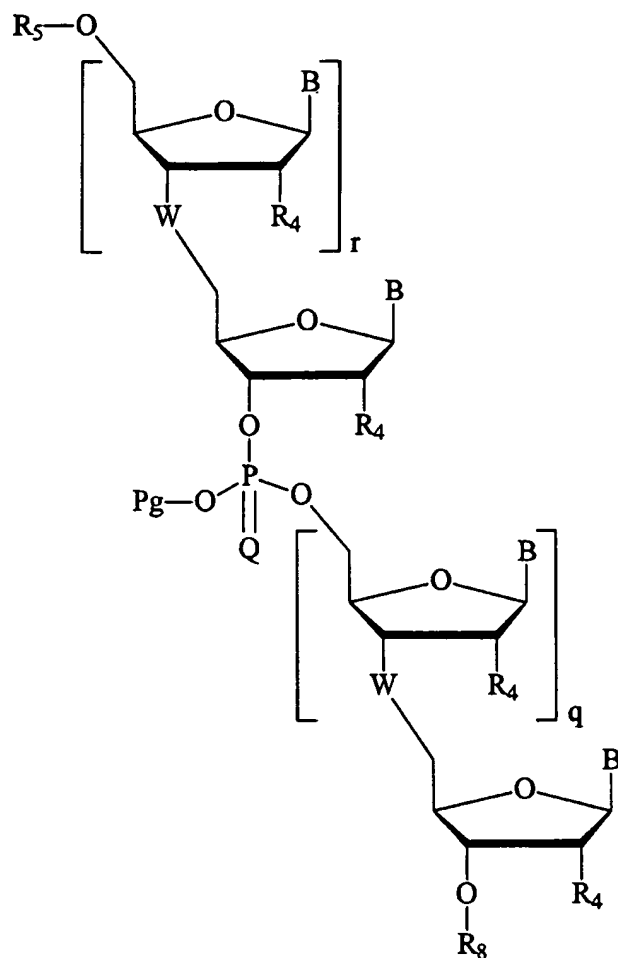
5 46. The method of claim 43 wherein r is 0.

47. The method of claim 43 wherein R_6 is diisopropylamino.

48. The method of claim 43 wherein Pg is $-CH_2CH_2CN$,
- $CH_2CH=CHCH_2CN$, para- $CH_2C_6H_4CH_2CN$, $-(CH_2)_{2-5}N(H)COCF_3$,
10 - $CH_2CH_2Si(C_6H_5)_2CH_3$, or $-CH_2CH_2N(CH_3)COCF_3$.

49. The method of claim 43 wherein Pg is $-CH_2CH_2CN$.

50. The method of claim 43 further comprising oxidizing or sulfurizing the compound of Formula XII to form a compound of Formula XIII:



XIII

where Q is O or S.

51. The method of claim 50 further comprising a capping
5 step.

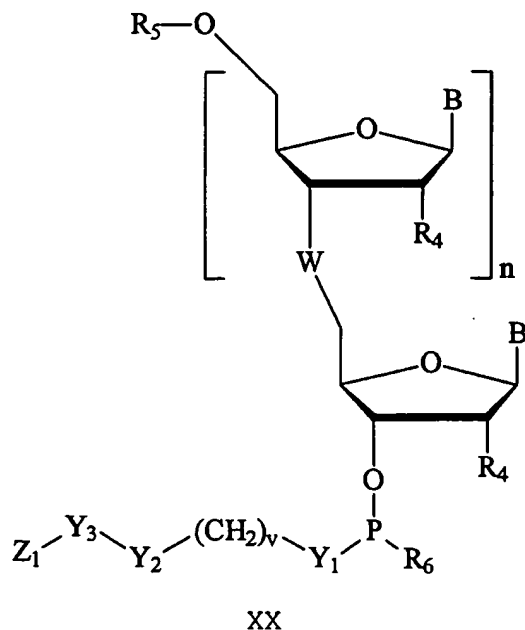
52. The method of claim 51 wherein the capping step is
performed prior to oxidation.

53. The method of claim 52 further comprising the step
of cleaving the oligomeric compound to produce a further
10 compound of Formula X.

54. A synthetic method comprising:

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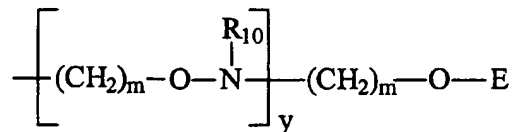
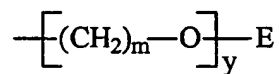
providing a compound of Formula XX:



wherein:

5 R_4 is H, F, O-R, S-R or N-R(R_{10});

R is H, a protecting group, or has one of the formulas:



where

10 each m is independently from 1 to 10;

y is from 0 to 10;

E is H, a hydroxyl protecting group, C_1 - C_{10} alkyl,

$N(R_{10})$ (R_{11}) or $N=C(R_{10})$ (R_{11}); substituted or unsubstituted

C_1 - C_{10} alkyl, C_2 - C_{10} alkenyl, C_2 - C_{10} alkynyl, wherein the

15 substitutions are selected from one or several halogen,

cyano, carboxy, hydroxy, nitro and mercapto residues; and

each R_{10} or R_{11} is, independently, H, substituted or unsubstituted C_1 - C_{10} alkyl, C_2 - C_{10} alkenyl, C_2 - C_{10} alkynyl, wherein the substitutions are selected from one or several halogen, cyano, carboxy, hydroxy, nitro and mercapto
5 residues; alkylthioalkyl, a nitrogen protecting group, or R_{10} and R_{11} , together, are a nitrogen protecting group or wherein R_{10} and R_{11} are joined in a ring structure that can include at least one heteroatom selected from N and O;

or R is $-\text{CH}_2-\text{CH}_2-\text{O}-\text{CH}_2-\text{CH}_2-\text{N}(\text{R}_{10})(\text{R}_{11})$;

10 R_5 is a hydroxyl protecting group;

Z_1 is aryl having 6 to about 14 carbon atoms or alkyl having from one to about six carbon atoms;

Y_1 is O or S;

Y_2 is O or S;

15 Y_3 is $\text{C}(=\text{O})$ or S;

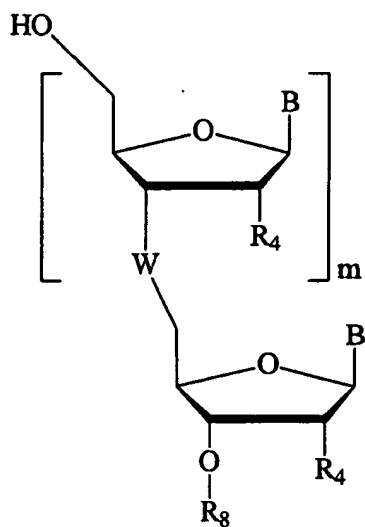
v is 2 to about 4;

B is a nucleobase;

R_6 is $-\text{N}(\text{R}_7)_2$ wherein R_7 is alkyl having from one to about six carbons; or R_7 is a heterocycloalkyl or
20 heterocycloalkenyl ring containing from 4 to 7 atoms, and having up to 3 heteroatoms selected from nitrogen, sulfur, and oxygen;

reacting said compound of Formula XX with a compound of Formula XXI:

- 140 -



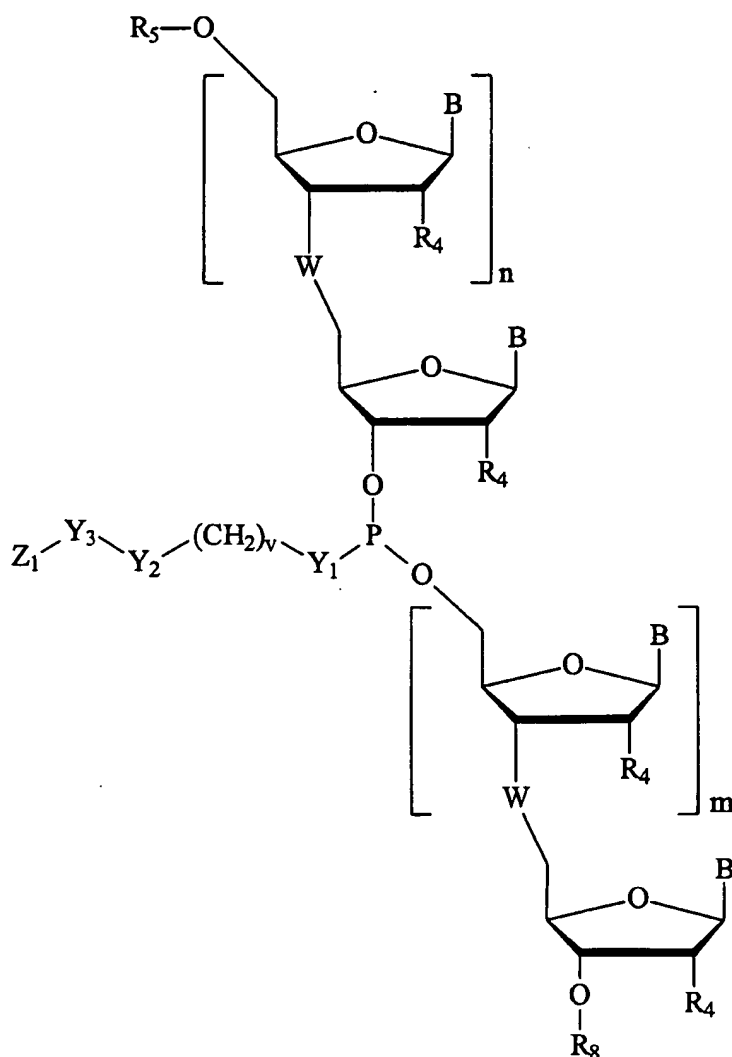
XXI

wherein:

R₈ is H, a hydroxyl protecting group, or a linker
5 connected to a solid support;

in the presence of an activator to form a compound of
Formula XXII:

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XXII

wherein said activator has the formula G^+U^- , where:

G^+ is selected from the group consisting of pyridinium,
 5 imidazolium, and benzimidazolium; and

U^- is selected from the group consisting of
 hexafluorophosphate, tetrafluoroborate, triflate,
 hydrochloride, trifluoroacetate, dichloroacetate, ^-O -mesyl,
 ^-O -tosyl, ^-Br , and ^-O -trifluorosulfonyl;

10 or said activator is a substituted imidazolium triflate.

55. The method of claim 54 wherein said activator is

imidazolium triflate.

56. The method of claim 55 wherein v is 2; and Y₃ is C(=O).

57. The method of claim 56 wherein Z is methyl, phenyl
5 or t-butyl.

58. The method of claim 57 wherein Z is t-butyl.

59. The method of claim 56 wherein n is 0.

60. The method of claim 56 wherein R₂ is a linker to a
10 solid support.

61. The method of claim 56 wherein Y₁ and Y₂ are each
O.

62. The method of claim 56 wherein Y₁ and Y₂ are each
S.

15 63. The method of claim 56 wherein Y₁ is O and Y₂ is S.

64. The method of claim 56 wherein each R₆ is
isopropyl.

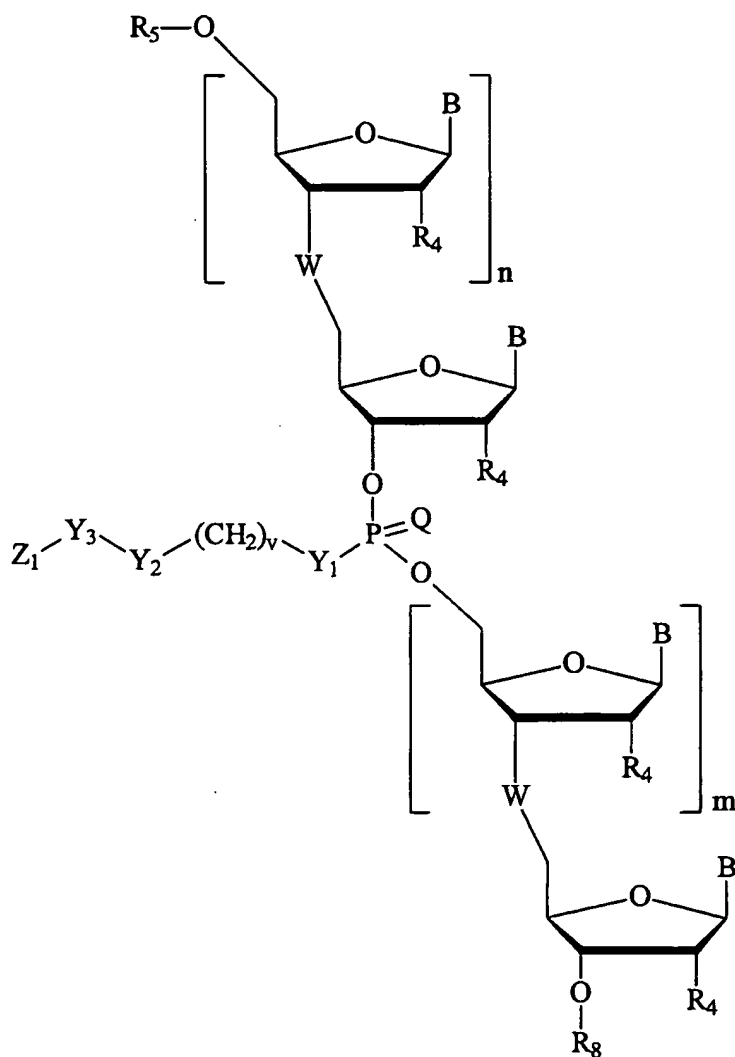
65. The method of claim 56 wherein n is 0; R₃ is H,
R₅ is diisopropylamino; Y₁ is O; Y₂ is S; and Z is methyl or
20 t-butyl.

66. The method of claim 65 wherein Z is t-butyl.

67. The method of claim 55 wherein each B is devoid of
exocyclic amine protection.

68. The method of claim 55 wherein W is an optionally protected phosphodiester, phosphorothioate, phosphorodithioate, or alkyl phosphonate internucleotide linkage.

- 5 69. The method of claim 55 further comprising oxidizing or sulfurizing the compounds of Formula XXII to form a compound of Formula XXIII:



10 where Q is O or S.

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70. The method of claim 69 further comprising a capping step.

71. The method of claim 70 wherein the capping step is performed prior to oxidation.

5 72. The method of claim 71 further comprising the step of cleaving the oligomeric compound to produce a further compound of Formula XXI.

73. The method of claim 43 wherein G^+ is pyridinium and U^- is hexafluorophosphate or tetrafluoroborate.

10 74. The method of claim 73 wherein U^- is hexafluorophosphate.

75. The method of claim 43 wherein G^+ is imidazolium or benzimidazolium and U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate, triflate,
15 hydrochloride, trifluoroacetate, dichloroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br , and ^-O -trifluorosulfonyl.

76. The method of claim 75 wherein G^+ is imidazolium or benzimidazolium and U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate, and triflate.

20 77. The method of claim 75 wherein G^+ is imidazolium or benzimidazolium and U^- is selected from the group consisting of hydrochloride, trifluoroacetate, dichloroacetate, ^-O -mesyl, ^-O -tosyl, ^-Br , and ^-O -trifluorosulfonyl.

78. The method of claim 43 wherein G^+ is imidazolium
25 and U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate, triflate, hydrochloride, trifluoroacetate, dichloroacetate, ^-O -mesyl,

O -tosyl, Br , and O -trifluorosulfonyl.

79. The method of claim 78 wherein U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate, and triflate.

5 80. The method of claim 78 wherein U^- is selected from the group consisting of hydrochloride, trifluoroacetate, dichloroacetate, O -mesyl, O -tosyl, Br , and O -trifluorosulfonyl.

81. The method of claim 43 wherein G^+ is
10 benzimidazolium and U^- is selected from the group consisting of hexafluorophosphate, tetrafluoroborate, triflate, hydrochloride, trifluoroacetate, dichloroacetate, O -mesyl, O -tosyl, Br , and O -trifluorosulfonyl.

82. The method of claim 81 wherein U^- is selected from
15 the group consisting of hexafluorophosphate, tetrafluoroborate, and triflate.

83. The method of claim 81 wherein U^- is selected from the group consisting of hydrochloride, trifluoroacetate, dichloroacetate, O -mesyl, O -tosyl, Br , and O -
20 trifluorosulfonyl.

84. The method of claim 43 wherein the activator is imidazolium triflate.

85. The method of claim 1 wherein B is devoid of exocyclic amine protection.

25 86. The method of claim 12 wherein B is devoid of exocyclic amine protection.

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87. The method of claim 18 wherein B is devoid of exocyclic amine protection.

88. The method of claim 24 wherein B is devoid of exocyclic amine protection.

5 89. The method of claim 29 wherein B is devoid of exocyclic amine protection.

90. The method of claim 43 wherein B is devoid of exocyclic amine protection.

10 91. The method of claim 84 wherein B is devoid of exocyclic amine protection.

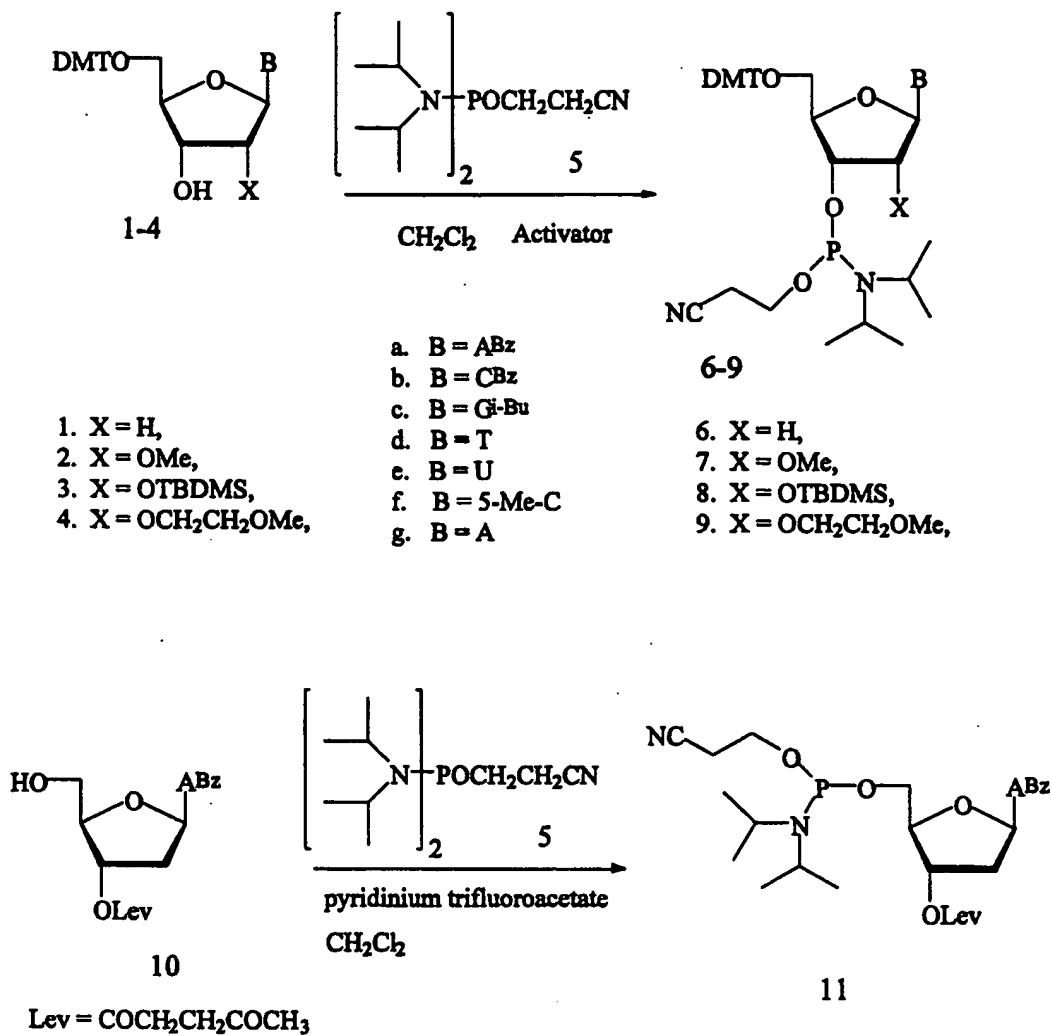
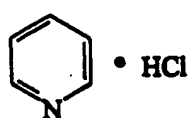


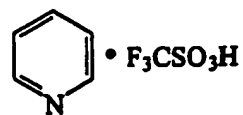
Figure 1



A, pyridine hydrochloride



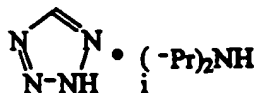
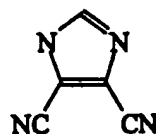
B, pyridinium trifluoroacetate



C, pyridinium triflate



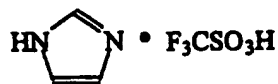
D, tetrazole

E, diisopropylammonium
tetrazolide

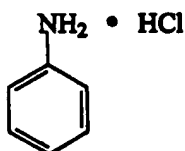
F, 4,5-dicyanoimidazole



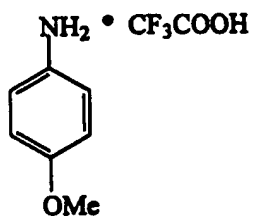
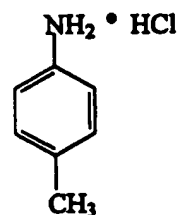
G, imidazole hydrochloride



H, imidazolium triflate

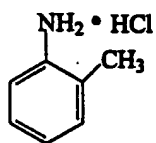


I, aniline hydrochloride

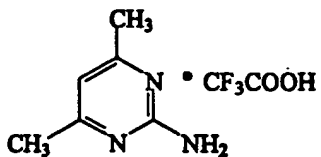
J, p-anisidine
trifluoroacetate

K, p-toluidine hydrochloride

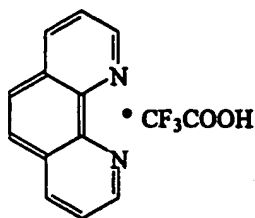
Figure 2



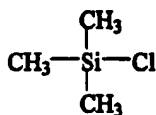
L, o-toluidine hydrochloride



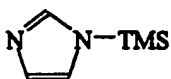
M, 2-amino-4,6-dimethylpyrimidine trifluoroacetate



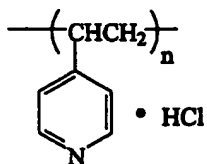
N, 1,10-phenanthroline trifluoroacetate



O, chlorotrimethylsilane



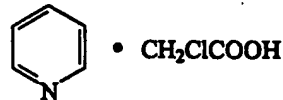
P, 1-(trimethylsilyl)imidazole



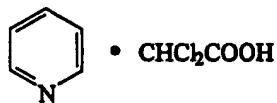
Q, poly(4-vinylpyridine hydrochloride)



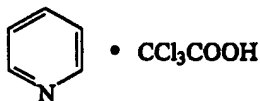
R, pyridinium acetate



S, pyridinium chloroacetate



T, pyridinium dichloroacetate



U, pyridinium trichloroacetate

Figure 3

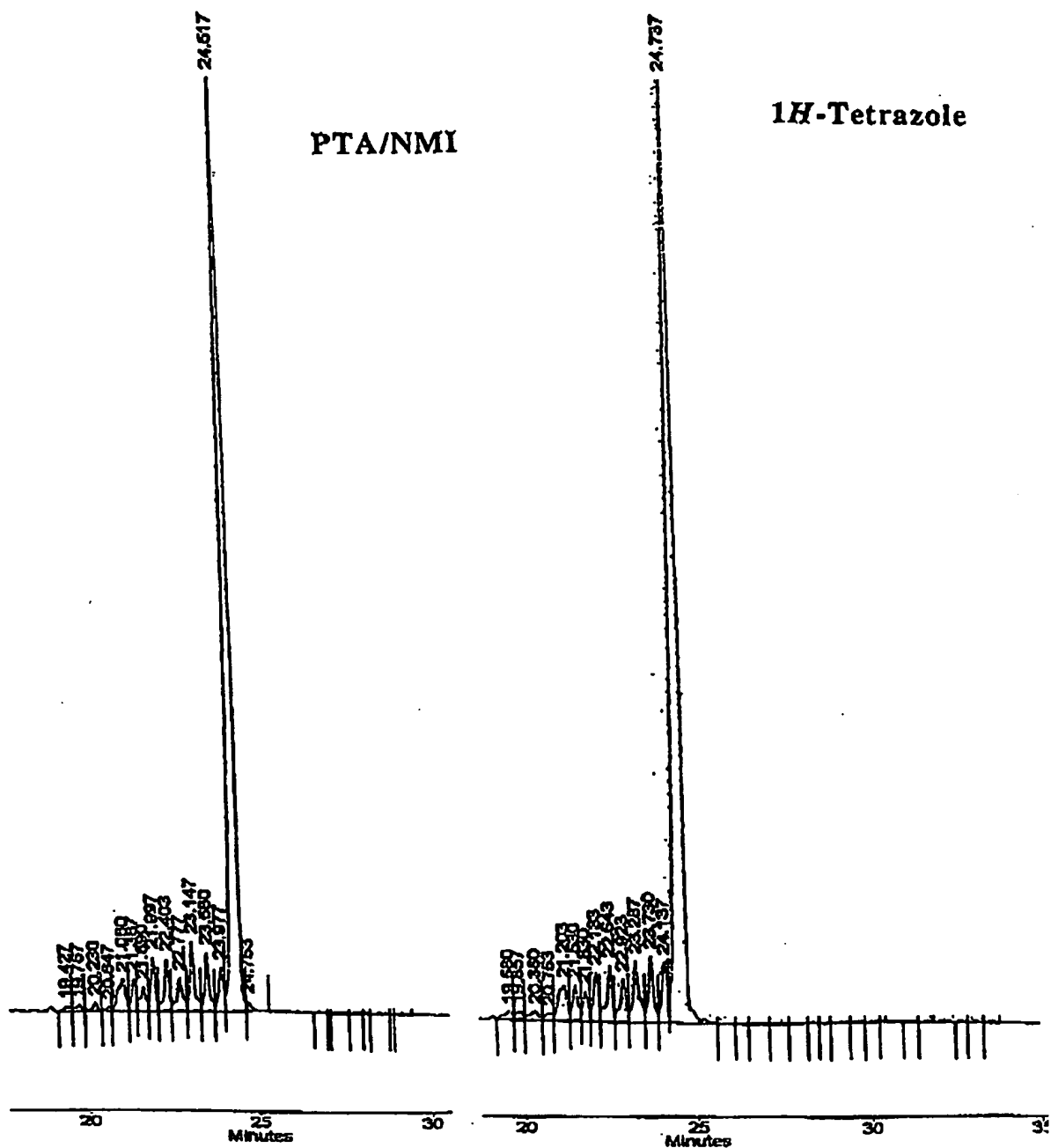


Figure 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/12251

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/00

US CL : 536/22.1, 23.1, 25.3, 25.33, 25.34, 25.4, 25.6

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/22.1, 23.1, 25.3, 25.33, 25.34, 25.4, 25.6

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS online

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,760,137 A (ROBINS et al) 26 July 1988, cols. 4, 5 and 6.	1-91
Y	US 4,997,926 A (HAERTLE et al) 05 March 1991, cols. 16-20.	1-91
Y	US 5,208,327 A (CHEN) 04 May 1993, see cols 3-7.	1-91



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

Z document member of the same patent family

Date of the actual completion of the international search

01 OCTOBER 1999

Date of mailing of the international search report

07 OCT 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
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